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THE RECOVERY OF TRANSMISSIVITY IN PASSIVE IRON WIRES AS A MODEL OF RECOVERY PROCESSES IN IRRITABLE LIVING SYSTEMS.

PART II.

BY RALPH S. LILLIE.

(From the Physiological Laboratory, Clark University, Worcester.)

(Received for publication, June 20, 1920.)

Theoretical Considerations.

The consideration of why transmission is only partial during the early period after repassivation must take account of a number of factors whose precise mode of action is not clear in all cases. It is evident that the return to the passive state implies redeposition of a continuous surface layer of oxidation product; it is also evident from the behavior of the newly passivated wire that when first deposited this layer is in a different chemical and physical condition from that which it afterwards attains when complete transmissivity is reestablished. The question is what kind of change occurs in the surface layer during the period of progressive recovery; *e.g.*, in 70 per cent HNO_3 during the first 7 or 8 minutes after the spontaneous return of passivity.

Several peculiarities are to be noted in the character of the activation wave at the successive intervals. There is an evident correlation between its speed and the distance which it travels. At first the local reaction spreads slowly and for only a short distance; at each successive trial the wave travels faster and advances farther; eventually it travels for an indefinite distance at a high speed (several hundred centimeters per second). It is also noticeable that an activation wave which comes spontaneously to rest after traveling some distance is visibly retarded through the last 2 or 3 cm. of its path (though not

evidently retarded earlier);[1] this retardation is more readily seen at low temperatures (3°C.), and in general in slowly moving than in rapidly moving waves.

It is well known that the advance of the wave is due to local cathodic reduction at the boundary region between the active and the passive areas of the metal, the passive area being cathodal.[2] The diagram (Fig. 1) illustrates the conditions. The active area is anodal and the adjoining passive area cathodal. The local intensity of the current passing between the solution and the passive metal at any point decreases with increase in the distance from the boundary line X, because of the increase in the electrical resistance of the circuit which includes the point under consideration. This resistance depends almost entirely upon the length and the specific electrical resistance

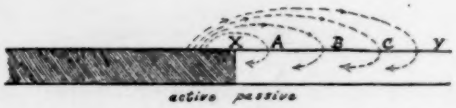

FIG. 1. Indicating the conditions of the local circuit at the boundary between the active and the passive areas; the direction of the current (positive stream) is indicated by the arrows. The active region (shaded) is anodal, the passive cathodal. See text.

of the column of electrolyte between the point in question and the boundary line. The local reducing action, which is a function of the local intensity of the current, thus decreases as the distance from the boundary increases, *i.e.* in the order A < B < C, and beyond a certain distance from the boundary (*e.g.* XY) it will be insufficient to

[1] Adrian (Adrian, E. D., *J. Physiol.*, 1914, xlviii, 53) finds no evidence of change in the velocity of the excitation wave in the nerve as it passes along a region of decrement; but, as he himself points out, his observations do not refer to the rate of conduction immediately before the extinction of the wave. He concludes that "if the rate of conduction is ever affected by the size of the disturbance it can be only when the disturbance is so small as to be on the verge of extinction." In the passive wire also the rate of transmission does not undergo evident retardation until immediately before extinction.

[2] *Cf.* Bennett, C. W., and Burnham, W. S., *J. Phys. Chem.*, 1917, xxi, 107. For the resemblances to protoplasmic transmission, *cf.* Lillie, R. S., *Am. J. Physiol.*, 1916, xli, 126; *Science*, 1918, xlviii, 51; 1919, l, 259, 416; *J. Phys. Chem.*, 1920, xxiv, 165.

effect reduction; during transmission, therefore, we are to assume that the removal of the film by cathodic reduction is continually in progress through a certain distance in advance of the boundary. The rate of this reducing action, and the distance from the boundary through which it is effective (*i.e.* the length of XY) are the two chief factors determining the speed of propagation.

It seems probable that in the decrement type of transmission the removal of the passivating film by cathodic reduction in the region XY adjoining the boundary is everywhere incomplete, and that the progressive decline in the traveling power of the activation wave is thus to be explained. In the case just considered, however, in which the local reduction is assumed to be complete, with consequent exposure of a free surface of metallic or "active" iron over the entire area XY, there will inevitably be complete transmission; *i.e.*, the activation wave, once set up, will travel through an indefinite distance, since then the area secondarily activated by the local current will have the same properties as the original active area; accordingly the current conditions at the boundary of this new active area will be the same as at the original area, and these conditions will be repeated at every new boundary as the active region extends. With exact repetition of the same reducing effect at the cathodal (*i.e.* passive) area adjoining each new boundary as it is formed, there is nothing to prevent transmission through an indefinite distance. In transmission with a decrement, however, it is evident that the chemical effect at each new boundary is somewhat less than before, as shown by the progressive decline in the traveling power of the activation wave. If, as the above hypothesis holds, the removal of the passivating film near the boundary is in this case only partial, this partially activated area will not reach the full anodal potential characteristic of a freely exposed iron surface; and the reducing effect at the passive area adjoining will be less than at the original active area; *i.e.*, there will be a progressive decline in the reducing power of the local circuit as the active area advances. Eventually, when the boundary of the active region has advanced for a certain distance from the starting point, the P.D. of the local circuit will fall below the critical value (*i.e.* the decomposition voltage) required for appreciable reduction; the advance of the active region will then cease, and the line of cessation will be

sharply defined. According to this hypothesis, the P.D. across the active-passive boundary decreases progressively as the activation wave passes along a region of decrement. When the rate of this decrease is gradual, there will be transmission through a relatively great distance before the wave comes to rest; when it is rapid, transmission will be correspondingly limited.

This hypothesis is also consistent with the fact that the return of transmissivity is more rapid in weak than in strong acid, its rate being approximately inversely proportional to the excess of concentration above a certain critical level (about 53 to 54 volumes per cent).[3] Two chief factors may be distinguished in the chemical part of the passivating process, *i.e.* in the reaction which reforms the passivating surface layer of oxide: (1) the general oxidative action of the solution, which is the more intense the higher the concentration of HNO_3; and (2) the electrochemical oxidative action at the anodal region of the local active-passive circuit. This anodal area, *i.e.* the active region of the metal, is automatically subjected to the oxidizing—and hence passivating—influence resident at every anode. The difference in the rate of repassivation is an index of the energy of the local oxidizing process; in acid of 50 volumes per cent (about 7.5 N) or lower this is not sufficient to rebuild a stable surface film, hence the metal continues to dissolve in this solution; in 55 per cent acid the oxidative action is intense enough to deposit a permanent film in 5 or 6 seconds (at 20°); in 60 per cent acid the rate of deposit (*i.e.* of repassivation) is several times more rapid; and at higher concentrations still more rapid. The low degree of transmissivity found immediately after repassivation suggests that the passivating film when first deposited is relatively thick (or relatively dense or otherwise resistant to alteration) in comparison with what it becomes later, when the state of transmissivity without decrement is reached. This view would imply that the quantity of passivating material deposited during the reaction

[3] The formula $\frac{C - 54}{t}$ = constant expresses fairly well the relation of the total recovery time to the concentration of the acid, where C is the concentration and t the recovery time. 53 to 54 per cent is the critical concentration; and the time required for the return of complete transmissivity is about proportional to the excess of concentration above this value.

is closely proportional to the excess of concentration of the acid above a certain critical limit (of about 53 to 54 per cent).[4] Hence in strong acid the film at its first deposition has a thickness (or a structure) such that the relative quantity removed by the cathodic reduction at the active-passive boundary when the metal is locally activated is less than in weaker acid. The decrement of transmission is hence from the first steeper in the strong acid, and a greater time is required for the removal of the surplus film material (by the solvent action of the acid) until it attains a degree of thinness permitting of rapid and complete removal under the influence of the local circuit.

The important fact from the point of view of the physiological comparison is that after the decrement stage has passed the local reaction is complete and does not admit of gradations. Transmissivity is then complete and the wire as a whole exhibits an "all or none" type of response. This stage is a permanent one, as long as the metal remains undisturbed in the acid, and apparently it corresponds to a condition of minimal thickness of film. Judging from the analogy with adsorption processes in general, it seems probable that at this stage the film is only 1 molecule in thickness.[5] It is evident that with a film of this thickness there would be no possibility of gradation in the local action; either it would be complete or would not occur. Uniform and complete action at the different areas of the surface in a transmitting wire would thus be necessitated, with the result that the activation wave would be transmitted for an unlimited distance with a uniform velocity whose exact degree would be determined by the local rate of reduction of the film.

These considerations lead to the conclusion that the condition reached by the passivating film when complete transmissivity is regained is one in which its material is spread out in a uniform layer of 1 molecule in thickness. In this respect it corresponds in structure to films of oil or fatty acid which have spread over the surface of water to an equilibrium stage, as in Langmuir's experiments.[6] The precise composition and structure of the film do not concern us at present;

[4] *Cf.* Lillie, R. S., *J. Gen. Physiol.*, 1920–21, iii, 119, Table II; and 125, Fig. 3.
[5] *Cf.* Freundlich, H., Kapillarchemie, Leipsic, 1909, 278.
[6] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

it is an oxidation product and is usually regarded as a higher oxide of iron.[7]

The question remains why the film persists for an indefinite period with unaltered properties, even in acid of a strength lower than that required to passivate. Why should not the film undergo still further solution and eventually lose its continuity and break down? In point of fact, the passivating film is unstable except in solutions possessing considerable oxidizing power;[8] its preservation thus appears to depend upon a more or less continuous process of slow oxidation; this process exercises what may be described as a regulative control over its local variations of composition or thickness. That this is the case is indicated by the general fact that passivity disappears spontaneously in pure water and in solutions of salts and other substances, except those having strong oxidizing properties. In the latter case there is evidence that local interruptions of the film (unless too extensive) are automatically repaired by local oxidative action. This may occur even in solutions whose oxidative properties are insufficiently intense to repassivate a completely activated wire. The case of nitric acid of specific gravity 1.20 or less will illustrate; in this solution an active wire continues to react until it is completely dissolved, but a passive wire remains unaltered indefinitely. I have already described how a film partially destroyed by brief immersion in M/1,200 NaCl is restored to its original state by brief immersion in 1.20 HNO_3.[9] A similar restoration after partial mechanical removal of the film is seen when the passive wire, while immersed in 1.20 HNO_3, is scraped locally with a piece of glass; in order to secure activation by this means several scrapes in rapid succession are as a rule required; if the interval between successive scrapes is lengthened this characteristic summation effect is less readily obtained. When a wire which is thus treated is connected through a voltmeter with another wire (*e.g.* of passive iron or platinum) serving as an indifferent electrode, there is seen at each scrape a slight temporary excursion of the needle

[7] Bennett and Burnham.[2] Bennett, C. W., and Burnham, W. S., *Tr. Am. Electrochem. Soc.*, 1916, xxix, 217. Langmuir (Langmuir, I., *Tr. Am. Electrochem. Soc.*, 1916, xxix, 260), however, has a somewhat different conception.

[8] For examples of this behavior *cf.* Lillie, R. S., *Science*, 1919, l, 259, 416.

[9] Lillie, R. S., *Science*, 1919, l, 259.

of the instrument, the scraped wire becoming more negative, indicating the formation of an anodal area. The range of this excursion is increased by a rapid succession of scrapes, and when a critical point is overpassed a propagated activation wave is started and the whole wire is activated. The reaction thus produced is permanent in acid of specific gravity 1.20 or lower; with higher concentrations it is temporary, as already described. The return of the potential to zero after its disturbance by the local destruction of the film evidently indicates the formation of a new film at the scraped area. Any local disruption of the film renders that area anodal, by exposing the underlying iron, and hence subjects it automatically to the anodal oxidizing influence. This at once reforms the film, unless the free metal is exposed over more than a certain critical area, in which case the active area *spreads* instead of becoming obliterated and the whole wire becomes active. Slight local disruptions, however, are at once repaired and the continuity of the film is in this manner automatically preserved. Hence the passive state is a stable one in solutions of sufficient oxidizing power.

It should be noted that this automatic tendency for the film to reform explains why the rapid removal of a considerable area of film is necessary for mechanical activation, and also the need for a brief interval between successive scrapes in the summation phenomena just described. It also explains the greater effectiveness of rapidly increasing as compared with slowly increasing currents in electrical activation.[10] Otherwise the film may be reformed as rapidly as it is removed, and the local effect remain insufficient to start a wave of activation.

We infer therefore that the condition of equilibrium which the passive metal reaches eventually when immersed in nitric acid is one in which a thin continuous layer of oxygen compound 1 molecule in thickness covers its entire surface. There is nothing to prevent a thicker layer from being thinned by solution until it reaches this limit, but any further removal is prevented by the automatic regulatory reaction just described. In this sense the preservation of the passivating film in an oxidizing solution is the expression of a "dy-

[10] Lillie, R. S., *Science*, 1918, xlviii, 57.

namic" rather than a "static" type of equilibrium; and there is seen an interesting analogy to the process by which a protoplasmic structure such as the plasma membrane maintains intact its structural continuity and dependent properties (semipermeability, polarizability, etc.) during life.

The brief duration of the period of activity in a wire activated in stronger solutions of nitric acid indicates a rapid reformation of the film, implying a correspondingly active local oxidation. The frequent failure of transmission in solutions stronger than 85 per cent (of 1.42 HNO_3) probably indicates a too intense oxidizing action, which interferes with the local reduction on which transmission depends. This view is confirmed by the fact, repeatedly verified throughout the present investigation, that mechanical activation is more readily induced in weaker than in stronger acid. In general, activation is favored by conditions that promote reduction (like making the metal cathodal) and hindered by conditions of the reverse type. This is illustrated by the following experiment. When two passive iron wires are placed side by side about 2 cm. apart in 60 per cent HNO_3 and connected through a key with the poles of a single Edison cell (about 0.9 volt), it is usually found, on closing the circuit, that the current is insufficient to activate the cathodal wire. During the flow of the current, however, this wire is more readily activated by scraping with a glass slide than while the current is not flowing; conversely, the anodal wire, during the flow of the current, is much more resistant to mechanical activation than before. But immediately after breaking the current the anodal wire becomes temporarily *more* reactive than normally—an effect due probably to its being now the cathode of the reverse or polarization current. Electrical activation is similarly modified by the passage of a constant current between a passive wire and the acid in which it is immersed; for example, while a current from five cells (about 4.5 volts) was passing through a passive wire anode immersed in 70 per cent HNO_3, touching with zinc caused a local activation which was conducted for only a few centimeters from the region of contact; after the current was broken the wave was transmitted as usual for the whole length of the wire. All the above effects indicate how closely the behavior of passive wires depends upon the condition of the surface film and upon the rate and character of the chemical

processes occurring at the surface of the metal. The parallelism of these physical effects to the phenomena of electrotonus in irritable living tissues is evident. Either facilitation or hindrance of the processes of physiological activation and conduction may result by altering the electrical polarization of the cell surface through a constant current, the effect varying according to the degree and orientation of the polarizing influence; and the above experiments show that the same is true of the film-covered metallic system.

It is remarkable how closely the behavior of passive iron wires during the period immediately following repassivation simulates that of partially narcotized or asphyxiated nerves, or of nerves in which some other kind of "block" is established. The "decrement" type of transmission characteristic of such wires is also shown by normal or completely transmissive passive wires under certain special conditions, *e.g.* in the neighborhood of a piece of platinum or other noble metal in close contact with the wire. Such contact invariably retards or prevents the passage of an activation wave started in another region. The explanation of this interference is simple; platinum is nobler in the electrochemical scale than passive iron; hence near the contact there exists a local circuit in which the iron is anodal, a condition which, as already described, interferes with activation and transmission. The following experiment will illustrate. If a piece of platinum wire or foil is placed across the middle region of a passive wire immersed in HNO_3 (of 60 to 80 per cent) and pressed into close contact by means of a glass rod, an activation wave started at one end of the wire is typically blocked at the region of contact and fails to enter the region beyond. The stoppage of the wave is not abrupt, but occurs with a progressive retardation which is plainly visible at a distance of 1 or 2 cm. from the contact. With an insufficiently close contact (*i.e.* too small a contact area) the block may not be complete; in such a case the activation wave is often observed to undergo marked retardation—at times almost stopping—at the contact; but after passing this region it regains its former speed, at first by a visible acceleration, and travels in the usual manner to the end of the wire. The parallel to a partial block in a nerve, due to local mechanical or chemical treatment, is evident; in this case also there is retardation in the altered region (or region of decrement), and, if the excitation

wave emerges from this region into the normal region beyond, it there regains its former velocity and other characteristics.[11] The conditions in the passive wire suggest that a block resulting from local injury or constriction in a nerve is due mainly to the influence of the local current (or injury current) between the altered and the unaltered regions—just as the platinum blocks the activation wave in a passive wire by interposing in its path a local circuit in which the iron is anodal and hence resistant to activation. In the metallic system partial compensation of the current of the approaching active-passive circuit by means of the current of the local Pt-Fe circuit is probably also a factor in the extinction of the wave. Analogous conditions may be presumed to exist in the living tissue.

A fully recovered passive wire exhibits an "all or none" type of behavior, in this respect also resembling a normal nerve; while any region of a passive wire which has only partially recovered is one in which an activation wave is conducted with a decrement. Such a local region of decrement may be produced at any desired position in a wire which is elsewhere completely transmissive by the simple method of touching the wire locally with a piece of zinc at an appropriate interval after the passage of a normal activation wave. A local region of temporary activity is thus produced, beyond whose limits the wire remains unaffected. This local region continues to conduct with a decrement at a time when the remainder of the wire has completely recovered. For example, in 65 per cent acid the recovery of complete transmissivity usually occupies between 4 and 5 minutes at 20°; at 2 minutes after a previous complete activation a new wave travels (on an average) for about 4 cm. from the point of contact. If at this time one touches the wire at a central point with zinc, there is formed a sharply defined newly activated region about 8 cm. in length, which 3 minutes later, at a time when the rest of the wire has recovered completely, still conducts with a decrement. An activation wave which is then started at one end of the wire will travel to this region of decrement and penetrate the latter (usually with a visible progressive retardation) for a variable distance of some centimeters. In a certain proportion of cases, if the time relations are

[11] Adrian, E. D., *J. Physiol.*, 1913, xlv, 389.

properly adjusted, the penetration is complete; and the wave, on emerging into the completely transmissive region beyond, recovers its former velocity and continues to the end of the wire. An activation wave will in this manner penetrate *two* regions of decrement separated by an interval of complete transmissivity, while it will be blocked by a single continuous region of a length equal to the sum of the other two. Such an experiment is closely comparable with Adrian's well known experiment in nerve.[11]

It is, however, difficult, on account of the variable behavior of different iron wires, to reproduce this result at will with the above method. The following procedure is more satisfactory and gives the

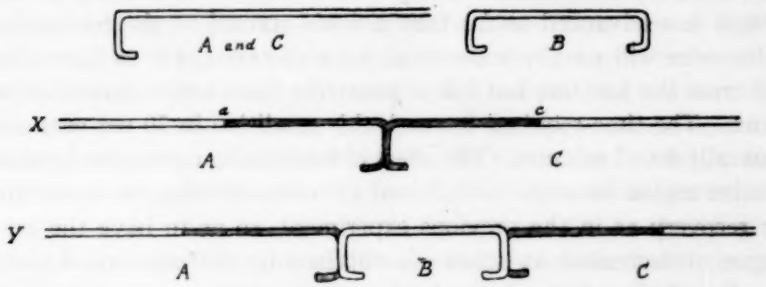

FIG. 2. Form and arrangement of wires in the experiment showing the difference in transmissivity between continuous and discontinuous decrement areas. See text.

result just described in a large proportion of cases. Instead of producing as above a region of decrement in a single continuous wire, three separate lengths of wire are used, bent in one plane as in the diagram (Fig. 2). Good contact between any two wires can be secured by resting one bent end across the other so that the right-angled pieces are in contact and the long portions in a straight line. In such an arrangement with two completely transmissive wires an activation wave travels from the end of one wire to the end of the other without appreciable retardation at the junction, and the two wires behave like a single wire of double length. The experiment is performed as follows. The three lengths of wire (first rubbed bright and clean) are passivated in the usual manner and transferred to a dish containing acid of 65 to 70 per cent strength; they are now in a com-

pletely transmissive condition and conduct activation rapidly from end to end. A region of decrement 4 cm. long is now made in each of the long wires A and C as indicated; this is done by pressing a piece of platinum foil against the wire at a point 4 cm. from the bent end and then touching this end with zinc; the wire is thus activated from its extremity to the edge of the platinum contact, and this recently activated stretch (the shaded area in Fig. 2) acts as a region of decrement for a period of several minutes. Wire B is used as a bridge between wires A and C as indicated; it is left unchanged; *i.e.*, is completely transmissive. When wires A and C are placed end to end, as in Fig. 2, X, there is a continuous region of decrement *ac* 8 cm. long; at a certain interval after the previous activation recovery in this stretch has advanced so far that a wave started at the free end of either wire will usually traverse *ac* for a distance of 6 to 7 cm.; *i.e.*, will cross the junction but fail to penetrate the whole region of decrement. The time required to reach this condition in 70 per cent acid is usually 6 to 7 minutes. The effect of interposing a completely transmissive region between wires A and C—after allowing the same time for recovery as in the previous experiment, so as to have the same degree of decrement as before—is obtained by uniting wires A and C not directly but through the bridge of completely transmissive wire B as indicated in Fig. 2, Y. In this case the wave, started at the free end of A, typically passes along the whole three wires to the extremity of C. In favorable experiments the retardation in the decrement region of A and the recovery of speed in the bridge B are plainly visible; the activation wave on entering C is then able to pass entirely through its decrement area to the end.

It is evident that the activation wave loses steadily in penetrative power—or what might be called intensity[12]—as it passes along the recently activated stretch or region of decrement; and regains this power when it enters the completely transmissive stretch beyond. This is the type of behavior also observed in a nerve fiber, as Adrian[11] has shown. In the wire the variations in transmissive power are

[12] Lucas points out that the only definite numerical measure, at present known, of the "intensity" of a nerve impulse is the distance which it can travel along a region of known decrement (Lucas, K., Conduction of the nervous impulse, New York, 1917, Chapter II).

dependent on variations in the condition of the surface film; and it is to be inferred, if the general conditions determining transmission are similar in the living system and the metallic model, that the same is true of the conducting protoplasmic strand or nerve fiber; *i.e.*, that the decrement-producing anesthetic acts by modifying the condition of the protoplasmic surface film.[13]

As Lucas and Adrian have pointed out, conduction with a decrement is undoubtedly a physiologically normal phenomenon in many regions of the central nervous system, as well as in the myoneural junctions and other nerve endings and synapses.[14] Whether a conducting element or combination of elements in the living organism transmits excitation in the "all or none" manner or with a decrement depends not only upon its special peculiarities of structure or organization but also upon its physiological condition—state of metabolism, fatigue, etc.—at the time. There is much independent evidence that protoplasmic excitation and transmission are in general dependent on local and transmitted alterations of the protoplasmic surface films or plasma membranes under the influence of the local bioelectric currents accompanying activity. If this is true, conditions changing the properties of these films must influence the whole behavior of the protoplasmic system. The phenomena of anesthesia in particular seem to afford many instances of this kind of correlation.[13]

In living animals the time relations of the recovery process in the different irritable cells and tissues vary widely, and they usually exhibit a close correlation with the time relations of the respective excitation processes. In most cases recovery is more rapid in the living tissue than in the passive iron model; yet this is not always the case. In the photoreceptors of Mollusca recovery may require several minutes, and in the smooth muscle of the mammalian ureter Engelmann found under some conditions imperfect transmission for 15 seconds or more after the passage of a contraction wave.[15] The

[13] The evidence that the anesthetic acts primarily upon the plasma membrane of cells is summarized in my review, The theory of anaesthesia (Lillie, R. S., *Biol. Bull.*, 1916, xxx, 352).

[14] Lucas, K., Conduction of the nervous impulse, New York, 1917, Chapters X and XIII.

[15] Engelmann, T. W., *Arch. ges. Physiol.*, 1869, ii, 271.

high velocity of the recovery process in highly irritable tissues like nerve, where the relative refractory period lasts for only a few thousandths of a second, must be referred to special peculiarities of structure and metabolism whose detailed nature is entirely unknown at present.

SUMMARY.

1. Passive iron (steel) wires, when activated after prolonged immersion in nitric acid of 55 to 90 per cent concentration (volumes per cent of HNO_3, specific gravity 1.42) revert spontaneously to the passive state, after a temporary reaction which is transmitted rapidly over the whole length of wire. The duration of this reaction at any region decreases rapidly with increase in the concentration above a certain critical limit of 52 to 54 per cent. In weaker acid (50 per cent and lower) the reaction continues uninterruptedly until all the metal is dissolved.

2. Immediately after this automatic repassivation the wire fails to transmit activation through more than a short distance (1 to 2 cm.); if left undisturbed in the acid it recovers by degrees its power of transmission (as measured by the distance traveled by an activation wave), at first slowly, then more rapidly; eventually, after an interval varying with the concentration of acid and the temperature, the activation wave is transmitted through an indefinite distance as before.

3. The return of complete transmissivity in 55 per cent acid occupies less than a minute (at 20°); in stronger acid it is more gradual, requiring in 90 per cent acid 20 minutes or more. This "complete recovery time" is nearly proportional to the excess of concentration of acid above the limiting value of 53 to 54 per cent.

4. In a given solution of acid the rate of recovery exhibits a temperature coefficient closely similar to that of most chemical reactions at this temperature (3–20°), and also to that of the rate of recovery (refractory period) of irritable living tissues after stimulation (Q_{10} = about 3).

5. Two definite phases are distinguishable in the recovery process: (1) the redeposition of the continuous passivating surface layer (of oxide or oxygen compound); and (2) the progressive change of the newly passivated wire from the state of incomplete to that of complete

transmissivity. The former phase is of brief duration and is indicated by a sudden change in the electrical potential of the wire, from that of active to that of passive iron; this phase is succeeded by the second and more prolonged period during which the passivating layer undergoes the progressive alteration associated with the recovery of transmissivity. This alteration appears to consist in a progressive thinning of the passivating film until a minimal thickness of (probably 1 molecule) is attained. Further thinning is prevented by local electrochemical oxidation.

6. The phenomena of partial or limited transmission during the second phase of the recovery process show a close correspondence with the phenomena of conduction with decrement in irritable living tissues such as nerve. Other analogies with the behavior of irritable tissues (threshold phenomena, distinction between "local" and "propagated" effects, summation, effects resembling electrotonus) are described.

A THEORY OF INJURY AND RECOVERY.

I. Experiments with Pure Salts.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, September 14, 1920.)

Previous researches[1] indicate that the process of death conforms to the laws of chemical dynamics and that further investigation of this subject may throw light on the fundamental mechanism of normal life processes. The present series of papers continues these studies and deals with the recovery of cells after exposure to toxic solutions.

When the marine alga *Laminaria* is transferred from sea water to a solution of sodium chloride (of the same conductivity as the sea water) its electrical resistance falls steadily until it reaches a stationary condition, which indicates death. If the tissue is replaced in sea water before this point is reached we observe that the resistance rises; this may be called recovery. In earlier stages of the death process recovery may be complete (*i.e.* the normal resistance may be regained) but this is not the case in the later stages. This is evident from Fig. 1, which shows the death curve in a solution of NaCl and recovery curves after various periods of exposure to the solution.[2]

If in place of sodium chloride we employ calcium chloride and various mixtures of these salts, varying the times of exposure, and sometimes transferring the plant from one of these solutions to another, instead of replacing it in sea water, a very complicated set of curves is obtained. It is of interest to find that these may be predicted

[1] Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533; *J. Gen. Physiol.*, 1920–21, iii, 15.

[2] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 15. The recovery curves rise to definite levels at which they may remain for days under favorable conditions. Often, however, there is a gradual decline which may be more rapid than that of the control.

with considerable accuracy by assuming that the electrical resistance is proportional to the amount of a substance in the cell which increases or decreases according to the relative proportion of salts in the external solution.

We assume that this substance (M) is formed and decomposed by a series of reactions[3] of the type[4]

$$O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$$

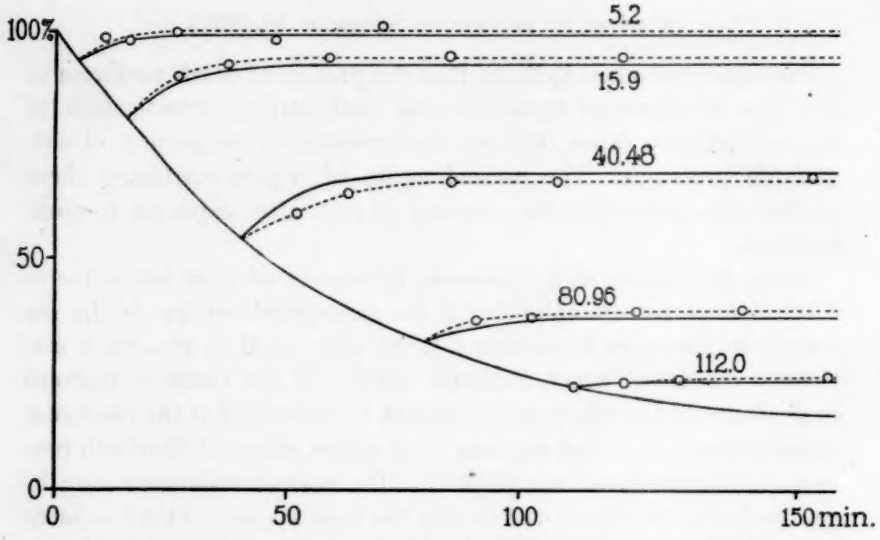

FIG. 1. Curves showing the fall of electrical resistance of *Laminaria agardhii* in 0.52 M NaCl (descending curve) and recovery in sea water (ascending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) of the solution of NaCl.

In the recovery curves the experimental results are shown by dotted lines, the calculated results by the unbroken lines (the curves are extended beyond the last observed point here shown because of later observations which are not shown in the figure).

The observed points represent the average of eight or more experiments; probable error of the mean less than 10 per cent of the mean.

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[3] These are regarded as irreversible or practically so.

[4] It is assumed that O is present in relatively large amount so that it may be regarded as practically constant despite the fact that it very slowly decomposes to furnish S, A, M, and B.

and that in sea water these processes are in equilibrium (so that the amount of M remains constant) but that when the tissue is transferred to a solution of sodium chloride M is decomposed faster than it is formed and hence the resistance falls. On replacing the tissue in sea water, M is formed more rapidly than it is decomposed and in consequence the resistance rises.

Let us assume that when the tissue is transferred from sea water to the solution of NaCl the reactions $O \rightarrow S \rightarrow A$ cease and that the velocity constant K_A of the reaction $A \rightarrow M$ increases from 0.0036 to 0.0180 while the velocity constant K_M of the reaction $M \rightarrow B$ increases from 0.1080 to 0.540. We may then calculate the resistance in the solution of NaCl after any length of exposure by means of the formula[5]

$$\text{Resistance} = 2{,}700\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 90\, e^{-K_M T} + 10 \qquad (1)$$

in which T is the time of exposure in minutes, and e is the basis of natural logarithms. 10 is added in the formula because the base line is taken as 10 (not as 0) for the reason that the resistance sinks to 10 (as shown in Fig. 1) when the tissue dies.

We assume that when the tissue is replaced in sea water the reactions $O \rightarrow S \rightarrow A$ recommence and that the values of K_A and K_M become 0.0036 and 0.1080 respectively, while the other velocity constants likewise acquire the values which they normally have in sea water. Under these conditions M will be formed faster than it is decomposed and the resistance will rise.

The fact that the rise does not reach as high a level after a long exposure as after a short one indicates that during the exposure O gradually diminishes; we assume that this takes place by the reactions

$$N \rightarrow O \rightarrow P$$

We likewise assume that during exposure to the solution of NaCl the amount of S changes by means of the reactions

$$R \rightarrow S \rightarrow T$$

and that on transferring to sea water S is rapidly converted into A.

[5] For an explanation of the formula see Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533. The constants 8.853 and 0.2951 are here multiplied by 305, becoming 2,700 and 90 respectively.

In order to calculate the rate of recovery we find by trial the most satisfactory values of the velocity constants. The values thus found are given in Table I.

TABLE I.

Velocity Constants.

Reaction.	Velocity constant.	Value at 15°C. in	
		NaCl	$CaCl_2$
$N \rightarrow O$	K_N	0.03	0.0045
$O \rightarrow P$	K_O	0.0297	0.004455
$R \rightarrow S$	K_R	0.04998	0.0145
$S \rightarrow T$	K_S	0.02856	0.007
$A \rightarrow M$	K_A	0.018	0.0018
$M \rightarrow B$	K_M	0.540	0.0295

As an example of the method of calculation we may take the case of tissue exposed for 15 minutes to a solution of 0.52 M NaCl at 17°C. The net[6] resistance in sea water at the start was 960 ohms; in the course of 15 minutes in the solution of NaCl it fell to 775 ohms, which is 80.69 per cent of the original resistance.[7] The fall of resistance is a little more rapid than in the "standard curve" obtained in a previous investigation.[8] If we assume that this is due to the difference in temperature (these measurements were made at 17°C. while those on which the standard curve is based were obtained at 15°C.) we may introduce a correction by multiplying the abscissa by the factor[9] 1.06, which makes it 15.9 minutes, and causes it to agree with the standard curve. All the abscissæ are multiplied by the same factor.[10] The

[6] The net resistance is that of the tissue itself, obtained by subtracting the resistance of the apparatus from the total resistance.

[7] For convenience all results are expressed as per cent of the original resistance.

[8] Osterhout, W. J. V., *J. Biol. Chem.*, 1917, xxxi, 585.

[9] This agrees closely with the temperature coefficient as determined in a previous investigation. *Cf.* Osterhout, W. J. V., *Biochem. Z.*, 1914, lxvii, 272.

[10] This procedure may displace the points on the curve so that where several curves are averaged it may be necessary to employ interpolation in order to average points on the same ordinate. In many cases curves were obtained by averaging the ordinates of death curves and recovery curves before multiplying by the factor.

effect of this is to make the process appear to proceed at 15 instead of at 17°C. If the difference between the two curves is due wholly to difference in temperature this introduces no error, and if the difference is due in part to other factors, the error, if any, is less than the usual experimental error.

The advantages of this procedure are that we can employ for our calculations the constants already obtained for the standard curve and also compare the theoretical curves which start from the same points. This procedure has therefore been followed throughout and the corrected results (*i.e.* the figures multiplied by a suitable factor) are employed in the following description.

When the tissue was replaced in sea water the resistance began to rise. At the end of 10 minutes it had risen from 80.69 to 92.57 per cent.[11] Since, however, the abscissæ of the death curve have been multiplied by 1.06 the same thing must be done for the recovery curve and in place of 10 minutes we must put 10.6 minutes. Proceeding in this manner we obtain the recovery curve which is labeled 15.9 in Fig. 1.

In order to calculate the course of the recovery curve we must consider the reactions which determine the amount of electrical resistance. When the tissue is placed in the solution of NaCl the reactions which occur are: (1) $A \rightarrow M \rightarrow B$; (2) $R \rightarrow S \rightarrow T$; and (3) $N \rightarrow O \rightarrow P$. Let us first consider the reactions $A \rightarrow M \rightarrow B$. The value of A in sea water is taken as 2,700 and that of M as 90. As explained in a former paper[1] the value of A will diminish during exposure to NaCl according to the formula

$$A\, e^{-K_A T} \qquad (2)$$

Since $K_A = 0.018$ (see Table I) the value of A after 15.9 minutes in sea water is

$$2{,}700\, e^{-(0.018)\ 15.9} = 2{,}027.96$$

The value of M at the end of 15.9 minutes is the observed resistance 80.69 less 10 (since the base line of the curve is not 0 but 10).

[11] In earlier experiments it was found that complete recovery was possible after the resistance had fallen to about 80 per cent. This was not the case in the present series; the difference may be due to differences in material or in technique. *Cf.* Osterhout, W. J. V., *Bot. Gaz.*, 1915, lix, 242.

On replacing the tissue in sea water, therefore, we start with M = 70.69 and A = 2,027.96, but this value of A is at once augmented by the conversion of S into A. In order to find the amount of this augmentation we must know the value of S.

During exposure to NaCl the reaction $R \rightarrow S \rightarrow T$ occurs. The value of S may be easily calculated by employing formula (1) and substituting the appropriate constants. We thus obtain

$$S = R\left(\frac{K_R}{K_S - K_R}\right)\left(e^{-K_R T} - e^{-K_S T}\right) + S_O\, e^{-K_S T} \qquad (3)$$

in which S_O denotes the value of S at the start of the reaction. The value of R in sea water is taken as 1,041.77 and that of S as 2.7. In the solution of NaCl the values of K_R (the velocity constant of the reaction $R \rightarrow S$) and K_S (the velocity constant of the reaction $S \rightarrow T$) are taken as 0.04998 and 0.02856 respectively (see Table I). Hence the value[12] of S at the end of 15.9 minutes is 447.26. When the tissue is replaced in sea water S is rapidly converted into A so that the total value of the latter becomes 447.26 + 2,027.96 = 2,475.22.

On replacing the tissue in sea water A = 2,475.22 and M = 70.69. The resistance after any given time T in sea water is obtained by modifying formula (1) which becomes

$$\text{Resistance} = 2{,}475.22\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 70.69\left(e^{-K_M T}\right) \qquad (4)$$

The velocity constants K_A and K_M have the normal values in sea water, 0.0036 and 0.1080 respectively. Hence the resistance at the end of 10.6 minutes is 87.44.

We must likewise remember that on replacing the tissue in sea water the reactions $O \rightarrow S \rightarrow A$ recommence and produce a certain amount of A; this breaks down to form M, which in turn decomposes. The resulting amount of M may be easily calculated. It will be recalled that in sea water all processes are so adjusted that the amount of M remains constant; it is evident that if the reactions $O \rightarrow S \rightarrow A$ were suddenly to stop, allowing $A \rightarrow M \rightarrow B$ to continue, the amount of M would diminish. At the start the total resistance is 100. If O

[12] In general the greater the rise in recovery the greater the value of S, while the greater the fall the less the value of S.

should stop producing this would diminish and we may call the loss of resistance L. Now if O were producing normally it would just replace this loss, so as to keep the resistance constant at 100: hence the amount produced from O in any given time will be equal to the loss L which would occur in that time if O were to stop producing.

When tissue is exposed to a solution of NaCl, O diminishes according to the scheme $N \rightarrow O \rightarrow P$. Assuming that at the start $N = 89.1$ and $O = 90$ we find[13] that the value of O after any given time (T) of exposure to a solution of NaCl may be obtained by changing the constants in formula (1) thus:

$$O = 89.1 \left(\frac{K_N}{K_O - K_N}\right)\left(e^{-K_N T} - e^{-K_O T}\right) - 90\, e^{-K_O T} + 10 \qquad (5)$$

in which K_N (the velocity constant of the reaction $N \rightarrow O$) and K_O (the velocity constant of the reaction $O \rightarrow P$) have the values 0.03 and 0.0297 respectively (see Table I).

We find by this formula that at the end of an exposure of 15.9 minutes the value of O is 92.57; hence it can produce only $(92.57 - 10) \div (100 - 10) = 0.917$ as much of M in any given time as it could produce if it were intact.[14] The amount it could produce, if intact, during recovery in sea water is easily found by subtracting from 100 the resistance obtained by means of formula (1), when $K_A = 0.0036$ and $K_M = 0.1080$ (these are the normal values in sea water). Using these values we find that at the end of 10.6 minutes the amount of resistance, as given by formula (1), would be 98.55. Hence the loss during that time would be $100 - 98.55 = 1.45$, which is the amount

[13] This value of O is assumed merely for convenience in calculation without reference to other assumed values. Its real value must be much greater than that of A but it is not necessary to assign any definite real value to it, since the only point of interest is to determine what per cent of O remains after any given time of exposure to sea water. It is assumed that in sea water any change in the amount of O is so small as to be negligible. This might be due to the fact that O is present in large amount and decomposes slowly or to the fact that it is formed as rapidly as it decomposes (by the reactions $N \rightarrow O \rightarrow P$).

[14] In other words, if S, T, and A were completely removed, O could raise the level of M to $100 - 10 = 90$ in the course of time. But if, for example, half of O is lost the remainder can raise the level of M to only one-half its former value; *i.e.*, to $45 + 10 = 55$.

O could produce in 10.6 minutes if intact: but as O has diminished to 0.917 times its original value it can produce in 10.6 minutes only (1.45) (0.917) = 1.33.

By adding this value to that obtained by formula (4) we find the resistance after 10.6 minutes in sea water to be 87.44 + 1.33 = 88.77.

In the same manner we may find the resistance at any given time after replacement in sea water. A series of values so obtained is given in Table II. It will be seen that they are in good agreement with the experimental values. The calculated and observed values are also plotted in Fig. 1, in which the abscissæ represent the time in the solution of NaCl plus the time of recovery in sea water (in the case just discussed this would amount to 15.9 + 10.6 = 26.5 minutes).

TABLE II.

Recovery in Sea Water after Exposure of 15.9 Minutes to 0.52 M NaCl.

Total time = time in sea water + 15.9.	Time in sea water.	Electrical resistance.	
		Observed.	Calculated.
min.	*min.*	*per cent*	*per cent*
26.5	10.6	89.10	88.77
37.1	21.2	93.21	91.34
58.3	42.4	93.99	92.43
84.8	68.9	94.48	92.52
121.9	106.0	94.20	92.55
164.3	148.4	93.81	92.55
545.9	530.0	94.00	92.57
863.9	848.0	93.87	92.57

Proceeding in this manner with different times of exposure we obtain the series of recovery curves shown in Fig. 1. The number attached to each curve denotes the time of exposure to the solution of NaCl. The observed results are plotted as dotted lines, the calculated values as unbroken lines.

It will be seen that the agreement is satisfactory throughout. In general the greater the number of experiments which were averaged to obtain the result the nearer it approached to the calculated curve.

Let us now consider the behavior of tissues transferred from a solution of 0.278 M $CaCl_2$ (which has the conductivity of sea water) to sea water. In such a solution the resistance rises and then falls. If

tissue is allowed to remain in the solution for a short time and is then replaced in sea water the resistance falls rapidly, as shown in Fig. 2. This fall of resistance may be regarded as analogous to the rise of resistance which occurs in the experiments with NaCl and the term recovery may be used in both cases. It is evident from the figure that, as the exposure to the solution of $CaCl_2$ lengthens, the level

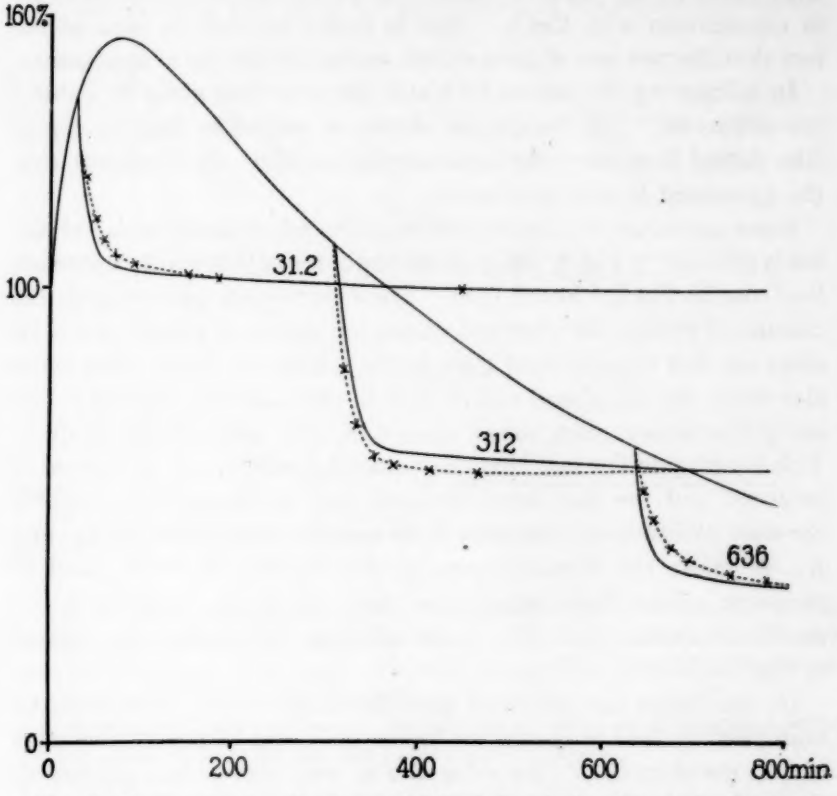

FIG. 2. Curves showing the rise and fall of electrical resistance in *Laminaria agardhii* in 0.278 M $CaCl_2$ (single curve which rises and falls) and recovery in sea water (descending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the solution of $CaCl_2$.

In the recovery curves the experimental results are shown by the dotted lines, the calculated results by the unbroken lines.

The observed points represent the average of eight or more experiments. Probable error of the mean less than 10 per cent of the mean.

which is reached as the result of recovery gets lower. This is precisely what happens in the experiments with NaCl. It would therefore appear as though the same mechanism of recovery were involved. If this is so the same method of calculation should enable us to predict recovery in both cases. This is found to be true. Using the same formulas which have already been employed in the experiments with NaCl we are able to predict the course of the curves obtained in experiments with $CaCl_2$. This is rather striking in view of the fact that the two sets of curves differ so fundamentally in appearance.

In calculating the curves for $CaCl_2$ the constants given in Table I are employed. The results are shown as unbroken lines in Fig. 2 (the dotted lines show the experimental results). It is evident that the agreement is very satisfactory.

Some assistance in picturing the reactions which occur during exposure is afforded by Fig. 3, which shows the curve of O in NaCl (unbroken line) and in $CaCl_2$ (dotted line). These curves are plotted from the calculated values; the observed values are shown as points; it will be observed that they lie fairly close to the calculated curve. The figure also shows the calculated values of S: in this case no observed values are given because such values cannot be very precisely determined. This is owing to the fact that the value of S affects only the speed of recovery (not the final level attained) and as the speed is variable the only satisfactory procedure is to assume such values of K_R and K_S as cause the closest approximation to the observed speed of recovery. When these values have been found the value of S can readily be calculated. The results of these calculations are plotted in Fig. 3.

In this figure the ordinates give the values of O: these must be multiplied by 6.75 to obtain the values of S. In all curves the value of S at the start is 2.7 (the value of S in sea water[15]); this appears on the ordinate in the figure as $2.7 \div 6.75 = 0.4$. The curves rise to a maximum and then fall to zero. The curves for O start at 100 and fall to 10 (since the base line is taken as 10, just as in the curve of M).

[15] The normal value of S in sea water is taken as 2.7 which is exceedingly small as compared with the amount of O. The amount of S which is produced from O in each unit of time is relatively large but S is so rapidly transferred into A that its amount in sea water never becomes greater than 2.7.

It will be observed that the rate of recovery is approximately the same in all cases; this applies to the experiments with $CaCl_2$ as well as with those in NaCl. In general it may be said that it usually requires about 60 minutes for the curve to complete nine-tenths of the total rise or fall which occurs in recovery.

With so large a number of constants it might seem possible to fit any sort of curve and hence the significance of the actual accomplish-

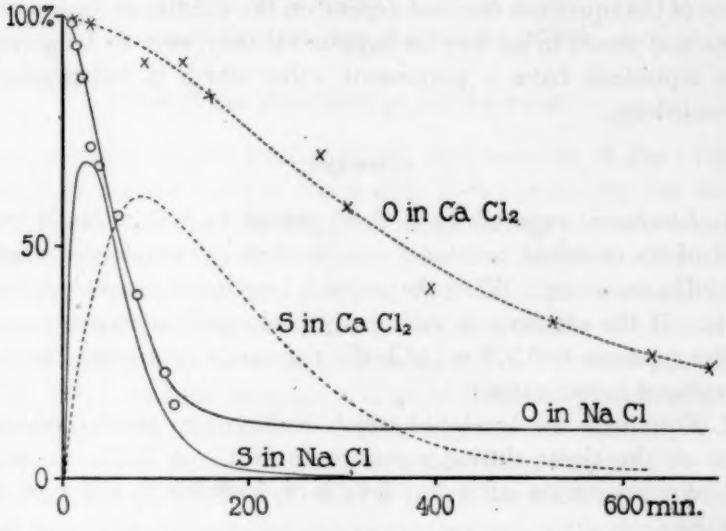

FIG. 3. Curves showing the values of *O* in NaCl (upper unbroken line) and in $CaCl_2$ (upper dotted line); also the values of *S* in NaCl (lower unbroken line) and in $CaCl_2$ (lower dotted line). The ordinates give the values of *O*; these must be multiplied by 6.75 to obtain the values of *S*.

The observed points represent the average of eight or more experiments; probable error of the mean less than 10 per cent of the mean.

ment might be lessened. This, however, is by no means the case. Moreover, the fixing of one or two constants affects the others in such a way as largely to determine the character of all the curves.

In the foregoing account many details are necessarily omitted, owing to lack of space. These, however, are not essential to the main purpose, which is to show how the process of injury and recovery may be analyzed and subjected to mathematical treatment. Starting

with certain assumptions we have formulated equations by means of which we can predict the behavior of the tissue. If the predictions are fairly accurate it is natural to infer that the assumptions are in accordance with the facts. It is evident from an examination of the figures that the equations enable us to predict with considerable accuracy the behavior of tissues in solutions of NaCl and $CaCl_2$, as well as the recovery curves after any length of exposure to either of these solutions. But we must not lose sight of the fact that the predictive value of the equations does not depend on the validity of these assumptions and would in no way be impaired if they were to be given up. The equations have a permanent value which is independent of assumptions.

SUMMARY.

1. *Laminaria* exposed for a short period to 0.52 M NaCl loses a part of its electrical resistance but recovers it completely when replaced in sea water. When the period is lengthened recovery is incomplete. If the exposure is sufficiently prolonged no recovery occurs. (After exposure to 0.278 M $CaCl_2$ the resistance falls when the tissue is replaced in sea water.)

2. Equations are developed which enable us to predict the resistance of the tissue during exposure to NaCl or $CaCl_2$ as well as the recovery curves after any length of exposure to either of these solutions.

LABYRINTH AND EQUILIBRIUM.

III. The Mechanism of the Static Functions of the Labyrinth.

By S. S. MAXWELL.

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(Received for publication, September 4, 1920.)

The Static Functions of the Ampullæ.

In a preceding paper[1] I have shown that removal of the otolith organs from the labyrinth of the dogfish does not destroy the static functions; that, on the contrary, the compensatory changes of position of the eyes and fins occur when the animal so operated is tilted out of the normal or primary position; and that if the animal is held in the abnormal position permanently the compensatory positions of the eyes and fins are also retained permanently. I have also pointed out that the stimuli for the maintenance of these compensatory positions do arise from the labyrinth; that they are not affected by the exclusion of other sensory stimuli; but that they disappear at once when the remaining parts of the labyrinth are destroyed. I also proved that rotations around the body axes excite the dynamic responses through changes of tension on the ampullæ due to the inertia of the vestibular contents, and not through the production of currents in the semicircular canals. I wish now to show that the same mechanism which excites the ampullæ to the exercise of their dynamic functions serves also to produce their static effects.

While an animal is undergoing rotation around a body axis there is brought about through inertia a displacement of the contents of the vestibule, and this displacement and the consequent change of tension acts as a stimulus. If on cessation of the movement of rotation the

[1] Maxwell, S. S., Labyrinth and equilibrium. I. A comparison of the effect of removal of the otolith organs and of the semicircular canals, *J. Gen. Physiol.*, 1919–20, ii, 123.

contents of the vestibule returned at once to their original position the stimulus would cease and the eyes would return to the primary position. If, however, the new position of the vestibular structures continued to exist after cessation of the movement the tension differences would also continue and the resulting stimuli would give rise to sustained forced position of the eyes; *i.e.*, to the static effect. The latter condition could exist in case the specific gravity of the utricular tissues is greater than that of the lymph. This I have found to be the case.

In most selachians the lymph of the vestibule is in free communication with the exterior sea water through the ductus endolymphaticus. It is reasonable therefore to expect that the density of the lymph would be practically equal to that of sea water. On this assumption I determined the relative weights of the membranous labyrinth and sea water by dropping small bits of utricle, ampullæ, and semicircular canals into a tall jar of sea water and saw that they all sank to the bottom. In order to be more certain, however, I succeeded in getting a sufficient amount of lymph from the ears of several fish killed at one time and dropped bits of the membranous labyrinth into it, with the result that they sank just as in sea water.

Since the membranous labyrinth and the lymph differ in specific gravity it is evident that when the membrane is displaced to a relatively lower position its weight will have the tendency to prevent its return to the original position in the cavity as long as the new body position is retained. I believe that this difference in weight, then, is the cause of the continued forced position in the absence of the otolith.

It has been frequently stated that the stimulation of an ampulla gives rise only to a momentary movement, not to a sustained forced position, and that therefore its function can be only dynamic and not static. I have found the contrary to be very definitely true. Sustained mechanical stimulation of an ampulla, even the ampulla of a horizontal canal, causes a sustained forced position of the two eyes; namely, a conjugate deviation to the side opposite to the stimulated ampulla. It is self-evident that in the ordinary functioning of the horizontal ampulla, when the rotation to which it responds is in a horizontal plane, no changed relation to gravity can occur and hence the reaction to rotation cannot continue after the rotation has ceased.

That the horizontal ampulla reacts to its normal stimulus by a response then is due only to its space relations, and not to a different kind of physiological function. Of course my experiments have demonstrated the ability of the other ampullæ to produce sustained static effects.

The Static Functions of the Otolith Organ.

In the dogfish and related forms it is comparatively easy to remove all the ampullæ and to see that in their absence both static and dynamic functions are retained by the remaining parts of the labyrinth; that is, compensatory movements and compensatory positions of the eyes and fins still occur in response to rotations in all planes except the horizontal. The removal of the large otolith of the sacculus has no effect whatever on these reactions; but if now, in addition, the small otolith of the recessus utriculi is removed the compensatory movements are at once abolished.

The original suggestion of Breuer that the otolith organs constitute the apparatus for the static functions of the labyrinth has been widely accepted. It has also been generally believed that the pressure of the otolith due to its weight is the stimulus which gives rise to the static function. If, for example, the animal is rotated to the right around its longitudinal axis and held in this new position, the pressure of the otoliths is shifted to the right and presumably the epithelium on the right side of the macula is now subjected to a relatively greater pressure, or even a previously unstimulated portion is now brought under pressure. In a previous paper[2] I was inclined to accept a similar explanation of the dynamic functioning of the otolith. In the light of new experiments to be reported below I am led to modify this view. I had, however, long ago given proof that in the compensatory movements of the horned toad (*Phrynosoma*) the exciting cause is not pressure, *per se*, but the torsion effect due to rotation.[3]

[2] Maxwell, S. S., Labyrinth and equilibrium. II. The mechanism of the dynamic functions of the labyrinth, *J. Gen. Physiol.*, 1919–20, ii, 349.

[3] Maxwell, S. S., On the exciting cause of compensatory movements, *Am. J. Physiol.*, 1911–12, xxix, 367.

Simple and plausible as Breuer's *a priori* conception appears no one has ever been able to confirm it by direct experiment. Lee,[4] for example, states that: "Stimulation of the otolithic parts of the ear has not been entirely satisfactory. The results were found exceedingly variable." Experiments by pressure on the otoliths or epithelium have usually been described as unconvincing or contradictory. As will be seen they are consistently contradictory to Breuer's views so commonly held.

I have now found that it is possible to stimulate mechanically the otolith organ of the recessus and to get results just as clear and consistent as those obtained from stimulation of the ampullæ. I quote the following record of an experiment.

"July 16, 1920. Large dogfish (*Galeus*).

Opened both ears and removed all six ampullæ.

Using a stiff bristle tipped with wax and the wax covered by a thin layer of absorbent cotton, applied pressure to various parts.

Right ear.

Pressed on right (lateral) side of otolith (of recessus utriculi); right eye depressed, left eye elevated.

Pressed on left side of otolith; left eye depressed, right eye elevated.

Repeated several times with uniform results. Otolith soon disintegrated: no more response.

Left ear.

Pressed on left side of otolith; left eye depressed, right eye elevated.

Pressed on right side (median) of otolith; right eye down, left eye up.

Repeated several times with same result."

Experiments made in this way gave fairly constant results, but it was not possible to repeat the observation many times without injury to the otolith organ. A new and very simple method was later found which permitted repetition of the stimulation many times before serious damage was done to the otolith and which gave absolutely constant results. The experiment is performed as follows:

A small mass of absorbent cotton is formed into a tiny cushion about the size of the otolith of the recessus and is cautiously placed on top of that otolith. The cotton is then grasped with the points of a fine

[4] Lee, F. S., A study of the sense of equilibrium in fishes. I, *J. Physiol.*, 1894, xv, 311.

forceps and gently moved to the right or left, forward or backward at will. I quote again from my notes:

"July 23, 1920. Large shovel-nosed ray (*Rhinobatus*).
Removed ampullæ from left ear.
Exposed small otolith (of recessus utriculi) and placed on it the pellet of cotton.
Movement of pellet to left caused depression of left eye and elevation of right eye.
Movement of pellet to right caused depression of right eye and elevation of left eye.
Movement of pellet forward caused both eyes to roll forward on their axes (anterior pole of each eye depressed and posterior pole elevated).
Movement of pellet backward caused both eyes to roll backward on their axes.
When pellet was moved to one side eyes moved in same sense.
When pellet was held to any side, the eye position was retained.
Removed the three ampullæ of the right ear.
Repeated the experiment on the right ear with exactly the same results.
Repeated a score or more of times with no noticeable diminution of the response.
Holding the pellet to any side held the eyes in the corresponding position."

I have repeated these experiments on dogfish, leopard sharks, and rays. The experiments on the ray (*Rhinobatus*) were particularly striking. This fish is broad and flat and usually remains on or near the bottom of the water. It is not apparently used to much turning over or tilting of the head up or down. Taken out of the water, or rotated in the water it does not show any of the compensatory movements in so marked a degree as does the dogfish. When, however, the stimulation was applied to the recessus as described above, the eye movements were extraordinarily vigorous, much more so than in response to rotation of the body of the uninjured animal. The eyes rolled right or left, forward or backward as if on actual mechanical axes manipulated by cords.

It will be seen from the above that pressure on the *right* side of the otolith of either ear produces the same eye movement which results as the compensatory motion to rotation of the body to the *left* around the longitudinal body axis; and that pressure on the *anterior* side of the otolith gives the same effect as tilting the head *upward*. In each case the response is precisely opposite to that which would be expected if the stimulation were produced by the pressure due to the weight of the otolith; for when the body is tilted to the right the weight of the

otolith must be shifted to the right, but the reaction of this rotation is elevation of the right eye and depression of the left eye. When pressure is applied directly to the right side of the otolith as in the experiments above described the opposite result is obtained; namely, depression of the right eye and elevation of the left. It must be then that the stimulation does not result from the direct effect of the pressure but from the shifting of the otolith; a displacement to the left is brought about by pressing on its right side under the conditions of the experiment, and a similar displacement to the left results from tilting the animal to the left. In other words, it is the *displacement* of the otolith, and not the pressure due to the weight of the otolith, which is the actual stimulus and it is the *direction* of the displacement which determines the direction of the compensatory movement in response to the stimulus.

The experiments described above show that the stimulus arising from the position of the otolith is not due to the pressure as such but to the relative tensions, and is in this particular exactly similar to what I had already found for the ampullæ. The stimulus which causes the forced position (static function) is, like the stimulus which causes the compensatory movement (dynamic function), due to relative differences of tension in the organ, rather than to localized stimulation of special portions. There is therefore no evidence for a specific difference in the mode of action of the various parts of an ampulla or a macula. The relative tensions appear to determine the proportionate nerve excitation for the associated muscle groups in a manner analogous perhaps to the effects of various degrees of tension in the lungs on the vagus endings. I have made no attempt to say just where these differences of tension take effect upon the nerve endings, nor to say what part, if any, the hair cells have in the process. The arrangement of the hair cells would seem admirable for the transmission of the effects of movement or pressure to the nerve endings, but I do not at present see how the matter can be subjected to the test of experiment.

A part of the experiments on which this paper is based were performed at the Scripps Institution for Biological Research. I take great pleasure in acknowledging my obligation to the Director, Professor W. E. Ritter, for his courtesy in placing the facilities of the Institution at my disposal.

THE ACTION OF INHIBITORY NERVES ON CARBON DIOXIDE PRODUCTION IN THE HEART GANGLION OF LIMULUS.

By WALTER E. GARREY.

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(Received for publication, September 18, 1920.)

While stimulation of nerves generally results in increased activity, the vagus has the opposite effect of diminishing the activity of the heart. The mechanism of this inhibition has not yet been explained. The author's recent experiments on the heart ganglion of *Limulus polyphemus*[1] have shown that stimulation of the inhibitory nerves of this heart results in a decrease in the production of CO_2 in the ganglion, while direct stimulation of the ganglion by electrical or mechanical or chemical means has the opposite result, namely an increase in CO_2 production in the heart ganglion, as will be shown in a later paper. This leaves no doubt that the action of the inhibitory nerves upon automatic ganglia is due to a decrease in those chemical processes in the ganglion which result in the formation of CO_2.

Methods.

The rate of CO_2 production was determined by the method previously described by the author.[2] Briefly, the ganglion, with its inhibitory nerve is dissected from the heart and immersed in 3 cc. of a

[1] The special suitability of the heart ganglion of *Limulus* arises from the fact that the heart may be isolated from the animal but beats for a day or more, likewise the ganglion may be dissected free from the heart and if connected with one segment alone will continue to deliver its impulses to the muscle for hours in sea water or in the solution used in this investigation. We are thus able to make our tests without concern about questions of changes of blood pressure, of vasomotor action, of chemical composition of the medium, or of the secondary action of other nerve supply which so complicate our experiments on the vertebrate nerve centers.

[2] Garrey, W. E., *J. Gen. Physiol.*, 1920–21, iii, 41, 49.

physiological saline solution consisting of 100 cc. of M/2 NaCl plus 2 cc. of M/2 $CaCl_2$ and containing phenolsulfonephthalein to indicate the hydrogen ion concentration.

We note the time required to produce the color change which indicates a change in the hydrogen ion concentration from the initial value pH 7.8 to 7.4. This gives a normal standard of the rate of development of CO_2 which can be compared with the rate at which the ganglion produces the same change while the inhibitory nerves are being stimulated.

Owing to the many failures to secure preparations of the ganglion with the inhibitory nerve uninjured and functioning, the following technique was adopted. Upon opening the dorsal carapace and pericardium the inhibitory nerves were identified by faradic stimulation; they were then secured distally with a fine silk ligature. The pericardium was removed from the whole heart and the cardiac ganglion, from its posterior end to the middle of the second segment, was dissected from the heart. This left functional connection of the ganglion with the first muscular segment which therefore continued to beat. The ganglion, free from all extraneous tissue, was now looped over tiny hooks on a slender glass rod which was cemented into the stopper of the indicator tube and the inhibitory nerves connected with it were laid across fine platinum wire electrodes passing through and cemented into the stopper. The nerves were again stimulated to determine whether they still inhibited the ganglion and stopped the beats of the first muscular segment. If the dissection has been successful it is only necessary to snip the nerve cord anterior to the point at which the inhibitory nerves enter to obtain the desired preparation of the ganglion mounted and ready for introduction into the tubes containing the solution with the indicator (phenolsulfonephthalein). We now determine the rate of the desired color change.[3]

[3] In a simpler preparation the ganglion alone was used and stimulation applied to its anterior end. This usually produces inhibition but stronger faradization may stimulate, so that the analysis of the results of the respiratory changes was sometimes problematical, therefore we have confined our published results to those obtained by the method described in the text.

Results.

The results of several typical experiments are summarized in Table I. The normal values for the time required to produce the change in pH are obtained before and after stimulating the inhibitory nerves and compared with the corresponding time while the stimulation is maintained. Differences in the time normally required by the different preparations to produce the change are due in part to differences in the size of the ganglia and in part to inherent differences in the

TABLE I.

Rate of CO_2 Production, before, during, and after Stimulation of Inhibitory Nerves.

	CO_2 production expressed in time required to produce color change.				
Experiment No.	1	2	3	4	5
	sec.	*sec.*	*sec.*	*sec.*	*sec.*
Normally. *a*	98	262	130	179	72
Normally. *b*	113	250	130	184	—
Normally. *c*	102	—	—	—	—
During inhibition	258	848	268	289	154
	—	—	242	—	—
After inhibition. *a*	197	546	154	201	78
After inhibition. *b*	124	314	—	—	75
After inhibition. *c*	120	286	126	180	—

intensity of the processes of CO_2 production. The results obtained by these experiments deal essentially with relative rates of CO_2 production, no attempt having been made to determine absolute values for the amount of CO_2 produced.

Analysis of the experiments leaves no doubt that inhibition of the automatic cardiac ganglion is a process involving a well marked depression of the CO_2 production in the ganglia (20 to 60 per cent of normal). The fact that the carbon dioxide production is only retarded and not completely suppressed is probably due to incomplete inhibition or to escape from inhibition toward the end of the period during which the determination is being made, since it has always been

noted that the rate at which the color change progressed was more rapid toward the end of a determination when the ganglion was presumably gradually escaping from inhibition.

In preparations showing a low initial rate of CO_2 production it is easy to depress this rate by inhibition for a much longer time than with vigorous preparations. In one experiment (not tabulated) no color change was noted at the end of 30 minutes of inhibitory nerve stimulation, although subsequently the normal rate of 5 minutes was restored. These observations are in complete harmony with what we know about inhibition of the contraction rate in this form.[4]

TABLE II.

Effect of Strength of Inhibiting Stimuli on Time For CO_2 Production.

Experiment No.	Normal (average).	Weak inhibition. Harvard coil. (13 to 10 cm.)	Strong inhibition. Harvard coil. (7 to 4 cm.)
	sec.	*sec.*	*sec.*
1	131	197	276
2	201	237	309
3	167	216	388
4	259	402	734
5	172	327	498
6	78	112	286
7	118	196	243

Since strong stimuli applied to the inhibitory nerves of the heart produce a greater slowing of the rate of heart beat than weak stimuli, experiments were made to see whether they produce a similar effect upon the rate of CO_2 production in the ganglia. Some typical experiments are given in Table II.

The results leave no doubt that the stronger inhibiting stimulation, which is known to produce a slower heart beat, also causes a greater depression of the rate of the chemical processes in the ganglia which give rise to the formation of CO_2.

If we view the entire process of inhibition we may represent the experimental results graphically as has been done in Fig. 1 which is constructed for four of the experiments in Table I. The graph shows

[4] Carlson, A. J., *Am. J. Physiol.*, 1905, xiii, 217.

that the drop in the rate of CO_2 production follows abruptly upon the incidence of inhibition but that the return to the normal after inhibition is a much more gradual process, indicating a persistence of the inhibitory state after the cessation of stimulation of the inhibitory nerves. The recovery to the normal rate of CO_2 production is slower the greater the degree of depression during inhibition.

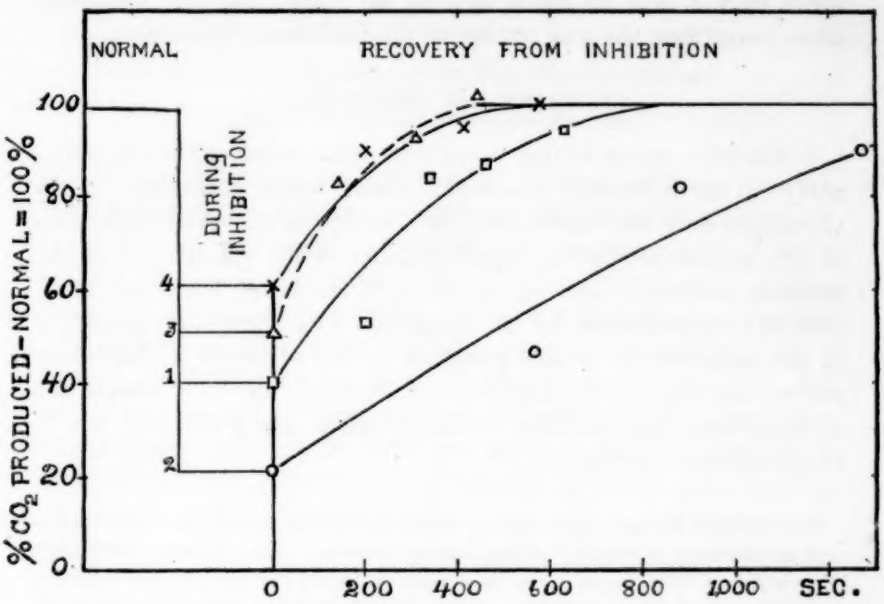

FIG. 1. Curves showing the percentage rate of CO_2 production compared with normal = 100 per cent. The numbers 1, 2, 3, and 4 correspond to the experiment numbers in Table I. The horizontal levels opposite these numbers show the average depression of CO_2 production during the period of inhibition which in each case continued for the time required to make the determination (*cf.* Table I). The readings for the curves of recovery are designated for each experiment by the character at the end of the inhibition period. Recovery time in seconds.

It is significant that the recovery process involves a return merely to the previous norm and that there is no subsequent increase in the rate of CO_2 production, no acceleration or after-augmentation, so that experimental evidence lends no support to the view that inhibition as exemplified in these nerve cells is accompanied by any con-

structive or anabolic processes. The latter processes are typical of arrest,[5] but mere cessation of action, such as occurs in blocking of impulses, is not to be confused with true inhibition which must be conceived as synonymous with depression of the chemical processes concerned in CO_2 production in the nerve cells. When this conception of inhibition is applied to other types of cells as well as to nerve cells, it will do much to clear up conflicting and confusing ideas concerning the true nature of the inhibitory mechanism.

SUMMARY AND CONCLUSIONS.

It has been shown in this paper that stimulation of the inhibitory nerves of the neurogenic heart of *Limulus*, which correspond to the vagus nerves of the vertebrate heart, results in a marked diminution of CO_2 production in the heart ganglion, while stimulation of the ganglion, leading to increased activity of the heart, leads also to increased CO_2 production by the ganglion. This shows that inhibition of the automaticity of this ganglion by the action of its inhibitory nerves consists, not in a process of blocking, but in a diminution of those chemical reactions in the ganglion cells which give rise to the production of CO_2.

[5] *Arrest* may be quite independent of inhibition and due to secondary causes such as blocking or removal of stimulating impulses. This is exemplified upon stimulation of the vagus fibers to the turtle heart; the auricles are inhibited (true depression), the ventricles merely arrested, and the latter only show the after-augmentation.

THE KINETICS OF INACTIVATION OF COMPLEMENT BY LIGHT.

By S. C. BROOKS.*

(From the Harvard School of Tropical Medicine, Boston.)

(Received for publication, August 29, 1920.)

In order to determine the course of an ordinary chemical reaction it is only necessary to mix the reagents and then to withdraw samples for analysis at appropriate intervals. But in the study of light reactions the case is far different, because the dimensions and transparency of the radiated material exercise a preponderant influence on the rate of reaction at any given intensity of illumination.

Since a great part of the difficulty encountered in studying the course of complement photoinactivation is due to the peculiar requirements imposed by these facts, which are often inadequately considered in connection with physiological problems, we shall begin with a discussion of these requirements, and of the means adopted to meet them.

I.

In the first place, since the intensity of the light reaching each part of the solution determines the rate of reaction at that point it is essential that the average intensity of the light reaching each part of the reaction mixture should remain constant during that part of the course studied. This is a condition which can be met only by special arrangements: either by having a cylindrical reaction chamber parallel to a relatively long "line source" of light, and withdrawing samples at intervals; or by exposing successive samples for a series of different periods under otherwise identical conditions. The latter alternative was adopted in these experiments.

* The experiments upon which this paper is based were done by the writer as Research Fellow in the Harvard School of Tropical Medicine.

In any case all parts of the radiated material must be exposed to essentially the same light intensity; otherwise the true order of the reaction cannot appear. In the case of serum, which is quite opaque to ultra-violet light, the part of the solution furthest from the light is shaded by the intervening serum. The opacity or absorption index of serum is so great that the back surface of a layer of complement diluted as much as practicable and only 0.2 mm. deep would still receive only 0.9 as much light (of the wave-lengths affecting complement) as the front surface. When the radiated material is a solution it can be vigorously stirred so that each portion is exposed to high and low light intensity alternately. In the case of a serum solution stirring is the simplest method of exposing all the solution to the same *average* light intensity.

Even with the two precautions indicated above, it is possible to determine the kinetics of a photochemical process only under certain restricted conditions which are not under the control of the experimenter, but inherent in the solution investigated. The existence of these conditions must be established by appropriate experiments before conclusions can be drawn as to the nature of the photochemical process itself. There are two points to be established: the absence of conditions causing progressive changes in the amount of effective light; and the negligibility of diffusion as a limiting factor in controlling the rate of reaction.

II.

If a reaction appears to be monomolecular, as complement photoinactivation does, it is always possible that this is the case simply because the reaction occurs with great velocity at some particular region, such as for example the walls of the containing vessel. The reaction will then appear to be monomolecular solely because the velocity of the reaction is limited by the amount of the substance arriving in the reaction field by diffusion, and, as the reaction proceeds, the rate of this diffusion decreases exponentially in accordance with Fick's Law.[1]

[1] Fick, A., *Ann. Phys. Chem.*, 1855, xciv, 59.

But diffusion is a process which is accelerated to a characteristic degree by an increase of temperature, and its presence as a limiting factor can be detected by conducting the reaction at different temperatures and noting how much its velocity is affected. The temperature coefficient, Q_{10}, for diffusion is 1.22 to 1.28, for "dark" chemical reactions usually 2.0 to 3.0 or above, and for light reactions 1.0 to 1.1. Some light reactions, notably those in which light does no work but acts merely as a catalyst, are exceptional in that they have higher coefficients.

Experiments were carried out early in the course of these studies to determine the temperature coefficient of the process of photoinactivation. A sample of complement was diluted to 2 per cent and divided into portions of 20 cc. each. These were exposed for different lengths of time at each of several different temperatures (0, 10, 20, 30, or 40°C.). The exposure was made in the manner previously described[2] except that the complement was maintained at the temperatures above 0° by partial immersion of the container in a water bath which was kept within 1° of the desired temperature. The calculation of reaction velocities is based on the assumption that the reaction is monomolecular. It will be apparent from data given further on in this paper that this is the most nearly accurate simple assumption which is possible (Table I).[3]

[2] Brooks, S. C., *J. Med. Research*, 1918, xxxviii, 345.

[3] The samples exposed at 30 and 40° are injured by heat as well as by light. The efficiencies given in Table I have been corrected as follows: the average rate of heat inactivation was calculated from the injury suffered by duplicate samples kept in the same water bath with each radiated sample for the corresponding length of time. Both photoinactivation (Section V of this paper), and thermoinactivation (Madsen, T., and Watabiki, T., *Overs. kong. danske Videnskab. Selskabs Forhandl.*, 1915, 125) follow the course of a monomolecular reaction and their observed velocities can be expressed as the velocity constant, k, of the monomolecular reaction isotherm, $k=\frac{1}{t}\log\frac{a}{a-x}$. By subtracting from k_O, the observed velocity when light and heat are both acting, the value k_H, observed when only heat is acting, we arrive at a value, k_L, which we may consider to be the component of the velocity due to the light alone. k_H was found to be 0.010 at 40 and 0.005 at 30°C. These values were subtracted from the k_O for each exposure, and from these values of k_L there were obtained (by the use of the reaction isotherm) corrected values of the efficiencies to be expected if light alone were to

In Fig. 1 the results of this experiment are graphically presented with the efficiencies of the different portions of complement plotted as ordinates against their respective periods of exposure as abscissæ. The smoothed curves drawn between the points for any one temperature allow us by interpolation to determine the exposure necessary at that temperature to reduce the efficiency of complement by any given amount. The rate of photoinactivation may be taken as inversely proportional to the time required to reduce the hemolytic power of complement to any selected per cent of efficiency. Calculation from such interpolated values of the average rate of inactivation

TABLE I.

The Efficiency of Samples of Complement Radiated at Different Temperatures for Different Intervals.

0°C.		10°C.		20°C.		30°C.		40°C.	
Exposure.	Efficiency.	Exposure.	Efficiency.	Exposure.	Efficiency.	Exposure.	Efficiency.	Exposure.	Efficiency.
min.	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*
4.0	65.0	4.0	—	4.0	63.8	3.5	64.7	2.5	62.4
7.0	41.8	7.0	52.5	7.0	53.2	5.0	56.3	3.5	62.6
10.0	42.8	10.0	41.8	10.0	38.0	8.0	45.3	5.0	47.3
14.0	27.8	14.0	26.2	14.0	25.9	12.0	29.7	8.0	39.8
21.0	12.0	21.0	11.0	21.0	—	17.0	15.3	12.0	28.4

to 60, 40, and 20 per cent efficiency shows that if the velocity at 0°C. is taken as 1.00 the velocities at 10, 20, 30, and 40°C. are 1.02, 1.09, 1.25, and 1.47, respectively. Hence the temperature coefficients for the four intervals studied are Q_{10} = 1.02, 1.07, 1.14, and 1.18. These values are probably accurate to the second significant figure only, so that although there appears to be a progressive increase in

act upon the complement. These values are given in Table I. The heat effect is negligible at temperatures of 20°C. or below, so that $k_O = k_L$ and no correction need be introduced at these temperatures.

These experiments were done previous to the perfection of the method for complement titration used for the remainder of the work described in this paper, and, as might be expected, there are occasionally large inaccuracies. These are obviously to be neglected in certain instances such as the 7 minute exposure at 0°C.

their value as higher temperatures are approached it would be premature to assume that such an increase would always be found. The mean value of Q_{10} is 1.10 and may be considered to be typical of the reaction. It is exactly that found in a preliminary experiment in which the rates of photoinactivation at 2 and at 38°C. were determined.

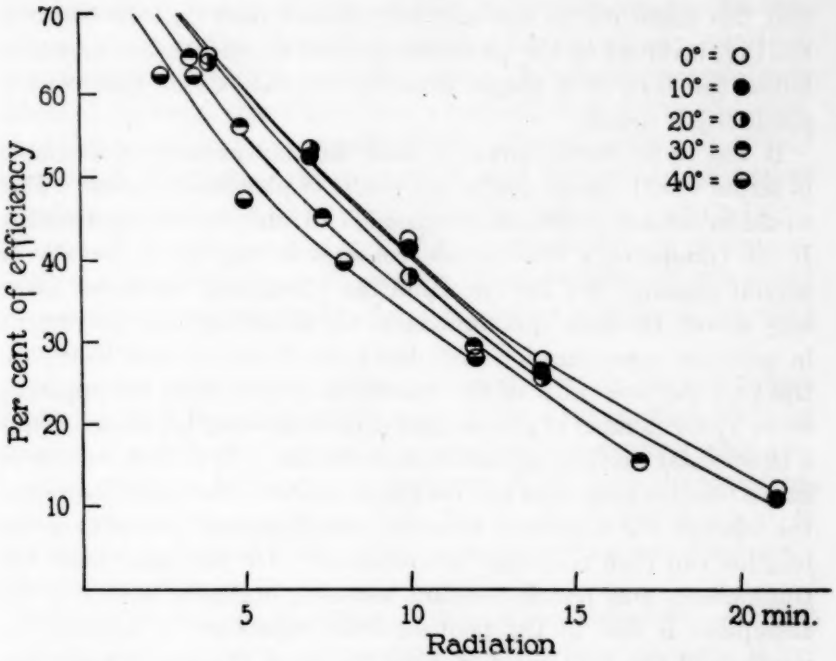

FIG. 1. Courses of the process of photoinactivation of complement at 0, 10, 20, 30, and 40°C. The ordinates represent efficiency in per cent of that of unradiated complement subjected to the same temperature conditions; and the abscissæ represent the time of exposure to light in minutes.

This value of the temperature coefficient for the process of photoinactivation is of particular significance because it is such as to allow us to neglect diffusion as a factor of any importance in determining the course of the reaction. For if diffusion were a limiting factor in the reaction the temperature coefficient of photoinactivation should be that of diffusion; *i.e.*, $Q_{10} = 1.28$. Instead of this it is 1.1 which is about the value which is to be expected in a photochemical reaction.

III.

But the progress of the light reaction is in turn affected by the amount of light absorbed, and by the photochemical efficiency of the absorbed light. We may assume that the latter remains constant; that is, that the same amount of light energy is needed at any stage of the process to make a single molecule react. It is conceivable that this might not be the case; but the very fact that the essential reaction involved in the photoinactivation of complement seems to follow the laws of a simple chemical reaction shows that such a possibility is remote.

It was to be feared, however, that the transparency of a sample of serum would change during the course of photoinactivation. This would have made it difficult or impossible to interpret the experiments. If the transparency does remain constant it may do so for any of several reasons. On the one hand the photolabile substance alone may absorb the light (photoproducts not absorbing) and be present in sufficient concentration and depth to absorb all the light. In this case the true order of the reaction is greater than the apparent order by the number of photosensitive molecules participating. Thus a bimolecular reaction appears monomolecular. It is obvious that as such a reaction progresses and the photosensitive substance disappears, the solution will ultimately transmit some light and the order of the reaction can then no longer be calculated. On the other hand the transparency may remain constant because a negligible portion of the absorption is due to the photosensitive substance or because the products of the light reaction have the same absorption coefficient as the reacting substances. In either of these cases the intensity of the light impinging upon the photosensitive molecules is constant, the amount of light absorbed may be assumed to vary as the concentration of absorbing molecules, *i.e.* Beer's Law,[4] and, if the solution is well stirred, the apparent order will be the same as the true order.

It can be shown that a layer of complement of the depth and concentration used in determining the course of photoinactivation absorbs practically all the effective light, and that the amount absorbed remains constant during the reaction; *i.e.*, that the photosensitive

[4] Beer, A., *Ann. Phys. Chem.*, 1852, lxxxvi, 78.

substance is constantly exposed to the same light intensity. Therefore if samples of complement are exposed to the light successively for varying periods, and well stirred during the exposure, the apparent order of the reaction will be the true order.

The transparency of complement was studied in the following way. A 5 per cent solution of fresh guinea pig serum in a physiologically balanced solution[5] was made up immediately after the serum was taken from the clot. Except during exposure to light the complement was kept at 0–1°C. The method of titrating the complement has been described in detail in a separate paper.[5] It consists essentially in allowing the complement to act on a suspension of specifically sensitized sheep erythrocytes suspended in the balanced solution previously mentioned, and noting the amount of each sample of complement required to produce a given amount of hemolysis in a given length of time. The relative efficiency of the various portions of complement was assumed to vary inversely as these amounts and was stated in per cent of the efficiency of an unradiated portion of the same complement.

It seemed desirable to determine approximately the absorption coefficient of solutions of complement of the concentration used; for from such observations it would be possible to determine what proportion of the incident effective light was absorbed in the apparatus used, and also, by analogy with the known absorption of serum, to determine the general region of the spectrum which was causing the photoinactivation.

To obtain such an approximate value for the absorption coefficient a 5 per cent solution of complement was placed in the inner of two concentric quartz test-tubes, and the balanced salt solution in the annular space between the two tubes; the space was 1.6 mm. wide. This set of tubes was then placed in a vertical position 9.5 cm. from a quartz mercury-vapor arc lamp and rotated on its long axis during an exposure of 3 minutes, which was exactly limited by the interposition of an opaque screen before and after radiation. Another portion of 5 per cent complement was then radiated in exactly the same manner except that the space between the two tubes (which

[5] Brooks, S. C., *J. Med. Research*, 1920, xli, 399.

were always the same ones) was occupied by 5 per cent complement. The ratio of the velocities of photoinactivation in the inner tube in the two cases is the same as the ratio of the intensities of the light incident on the inner tube in the two cases, or in other words, since the salt solution absorbs a negligible amount of light, as the ratio of incident to emergent light when the complement is in a layer 1.6

TABLE II.

The Efficiencies in Per Cent and Velocities of Inactivation of Paired Samples of Complement, One Sample of Each Pair Being Radiated through a Layer of Complement and the Other through a Similar Layer of Balanced Salt Solution.

Sample.	Efficiency after exposure.	Velocity of inactivation.	Absorption coefficient.*
	per cent		
1*a*	87.4	0.020	74.8
1*b*	92.8	0.011	
2*a*	83.9	0.026	144.8
2*b*	94.9	0.008	
3*a*	91.5	0.0127	167.0
3*b*	96.4	0.0033	
4*a*	79.8	0.0327	54.1
4*b*	86.5	0.0212	
Average			110.2

* In the last column are given the absorption coefficients, μ, calculated from the formula $\mu = \frac{1}{dc} . ln \frac{I_0}{I}$; where d = thickness of the absorbing layer in centimeters, c = concentration of absorbing substance, and I_0 and I incident and emergent light intensities.

mm. deep. Four trials were made and the corresponding absorption coefficients calculated as shown in Table II. In calculating the velocities of reaction, as in the case of Table I, the reaction is supposed to follow a monomolecular course. The rather great divergence between the four observed values is probably explained by variations in the amount of pigment in the different samples of serum.

The average of the four values of the absorption coefficient is 110.2 and may properly be considered to be of the true order of magnitude. This is about the absorption to be expected if light of a wave-length of about 2530 Ångström units were acting, while light of shorter wave-lengths is much more strongly absorbed.[6] The mercury arc emits little light of wave-lengths intermediate between that of a line at about 2530 Ångström units and that of about 2930 Ångström units, and light of wave-length greater than about 2900 Ångström units has little or no effect on complement.[7]

We may conclude that the principal effect of light on complement is due to ultra-violet light having a wave-length of about 2530 Ångström units; and that complement in layers of the depth used in the experiments on the course of photoinactivation absorbs a large part of the available light. For example, the most transparent sample, having an absorption coefficient of 54.1, would absorb nearly 95 per cent, the most opaque sample more than 99 per cent, of the incident light of the effective wave-length.

IV.

It was necessary, as has been pointed out in the preceding section, to determine whether or not there were changes in the transparency of complement during the progress of photoinactivation. To settle this point successive portions of complement were exposed for exactly equal lengths of time in the inner of the two concentric quartz tubes arranged as described above. In the space between the two tubes was placed a portion of the same solution of complement; this portion was left throughout the series of exposures. The total time of radiation of this sample varied in different experiments between 18 and $25\frac{1}{2}$ minutes, and was long enough to reduce the efficiency of the complement to less than that attained in any of the experiments on the course of photoinactivation. Control experiments were conducted which were similar except that the outer space was filled with distilled water or with complement which had been completely inactivated

[6] Henri, V., Henri, Mme. V., and Wurmser, R., *Compt. rend. Soc. biol.*, 1912, lxxiii, 319.

[7] Bovie, W. T., *J. Med. Research*, 1918, xxxviii, 335.

by long exposure to the light before the first sample was placed in the inner tube. If there were changes in transparency during photoinactivation successive samples should vary in their efficiency, and this variation should be greater than that displayed by successive samples in the control experiments. Examination of Table III will show that such is not the case and that therefore the transparency of the complement solutions remains constant during photoinactivation. In no case does the probable error of a single reading exceed

TABLE III.

The Variation in Efficiency of Portions of Complement Successively Exposed to Light in the Inner of Two Concentric Quartz Tubes for Equal Lengths of Time.

	Space between tubes filled with							
	Fresh complement.					Previously photoinactivated complement.		
Experiment No.	79	84	85	94	95	82	92	93
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Efficiency, in per cent, of successive portions.	84.7	96.7	94.0	95.8	85.0	86.7	90.9	95.2
	85.0	95.4	96.1	94.6	88.1	88.0	93.2	94.7
	86.2	97.8	96.3	95.2	86.0	89.1	93.8	94.2
	85.3	92.5	—	93.1	88.8	87.2	93.2	95.8
	85.1	95.5	—	89.9	85.9	87.1	91.5	95.9
	85.7	—	—	91.8	89.1	—	93.8	—
Mean efficiency.	85.3	95.6	95.5	93.8	87.5	87.6	92.7	95.2
Probable error of a single determination.	0.4	1.3	0.9	1.1	1.2	0.6	0.8	0.6
Mean probable error.	0.96					0.67		

that to be expected as a result of errors inherent in the method of titration, and although the mean calculated probable error is slightly less in the controls the difference cannot be considered significant.

We may sum up all these preliminary experiments with the statement that the apparent order of the reaction is the true order, because the effective light (probably that having a wave-length of 2536 Ångström units) is absorbed by complement in the same degree during all stages of photoinactivation. With the arrangement used

in the experiments about to be described, more than 95 per cent of the incident light of the effective frequency is absorbed by the serum solution.

The time curves of complement photoinactivation which are given in the following section are therefore susceptible to analysis in terms of chemical kinetics; if the process follows the course of any simple chemical reaction it may be interpreted in terms of the number of kinds of molecules participating in the reaction.

V.

The course of complement photoinactivation was determined by radiating a number of portions of the same lot of complement for different periods of time, and plotting their efficiencies as ordinates against their respective periods of exposure as abscissæ. The curve connecting these points is assumed to represent the progressive change in efficiency which would occur in a single sample of complement. It was important that there should be no doubt about the fact that the amount of light action was characteristic of the particular serum used, and not simply an amount which might vary according to accidental differences between different exposures. To establish the fact that a given exposure would always cause the same amount of injury to a given complement solution three portions of complement were exposed for 10 minutes each, and then titrated. Their efficiencies were found to be 56.8, 54.4, and 54.7 per cent respectively; the mean efficiency was 55.3 per cent and the probable error of a single determination was 0.87 per cent which is 1.6 per cent of the mean value. This may be attributed entirely to errors in titration. Further examples of constant amounts of injury caused by short equal exposures may be found in Table III above.

Table IV and Fig. 2 present the course followed by the process of photoinactivation of eight different sera. They include all the dependable data obtained, a large number of the earlier experiments having to be discarded because of the difficulty of so arranging the titration as to include for each partially inactivated sample just those dilutions needed to yield a complete "titration curve." While it is probable that other types of inactivation curves might be encountered in the

TABLE IV.

Efficiency of Portions of Eight Complement Solutions Radiated for Different Periods, and the Corresponding Values of the Term $k = \frac{1}{t} \log \frac{a}{a-x}$.

Experiment No.	Data.	Observed values after exposures of duration indicated.						
61	Exposure, *min*	0.75	1.5	2.58	4.13	6.0	9.08	15.0
	Efficiency, *per cent*	85.9	83.3	78.0	74.1	62.4	50.8	36.3
	k	0.089	0.053	0.042	0.315	0.034	0.032	0.029
62	Exposure, *min*	0.75	2.5	4.0	6.0	9.0	13.0	22.0
	Efficiency, *per cent*	94.2	76.2	72.0	67.9	46.7	41.1	23.7
	k	0.035	0.047	0.036	0.028	0.037	0.03	0.028
64	Exposure, *min*	0.75	2.5	4.0	6.08	9.0	0.3	21.0
	Efficiency, *per cent*	97.4	91.0	83.7	76.3	66.7	55.4	33.2
	k	0.016	0.016	0.019	0.019	0.02	0.02	0.023
67	Exposure, *min*	1.0	2.5	4.0	6.0	9.0	14.0	20.75
	Efficiency, *per cent*	93.2	84.5	76.3	66.6	54.3	39.0	19.7
	k	0.031	0.03	0.03	0.03	0.03	0.029	0.034
69	Exposure, *min*	0.55	1.0	2.5	4.0	7.0	11.0	18.0
	Efficiency, *per cent*	110.7	114.6	86.4	63±*	54.9*	52.5	28.9
	k	—	—	0.026	0.048	0.037	0.026	0.03
70	Exposure, *min*	0.67	1.33	3.0	6.0	10.0	16.0	24.0
	Efficiency, *per cent*	90.3	86.4	75.4	71.3	52.5	31.8	15.7
	k	0.067	0.048	0.041	0.025	0.028	0.031	0.033
71	Exposure, *min*	1.0	2.0	3.5	6.0	10.0	16.0	25.0
	Efficiency, *per cent*	97.8	91.0	82.4	71.5	52.4	37.7	25.6
	k	0.01	0.021	0.024	0.024	0.028	0.026	0.024
72	Exposure, *min*	0.5	1.0	2.0	4.0	7.0	10.0	18.0
	Efficiency, per *cent*	95.7	90.9	85.6	74.0	57.6	43.6	23.6
	k	0.04	0.042	0.034	0.033	0.034	0.036	0.034
Mean.	Exposure, *min*	1.0	2.0	4.0	7.0	10.0	15.0	20.0
	k	0.026	0.032	0.03	0.028	0.03	0.028	0.028

* Doubtful.

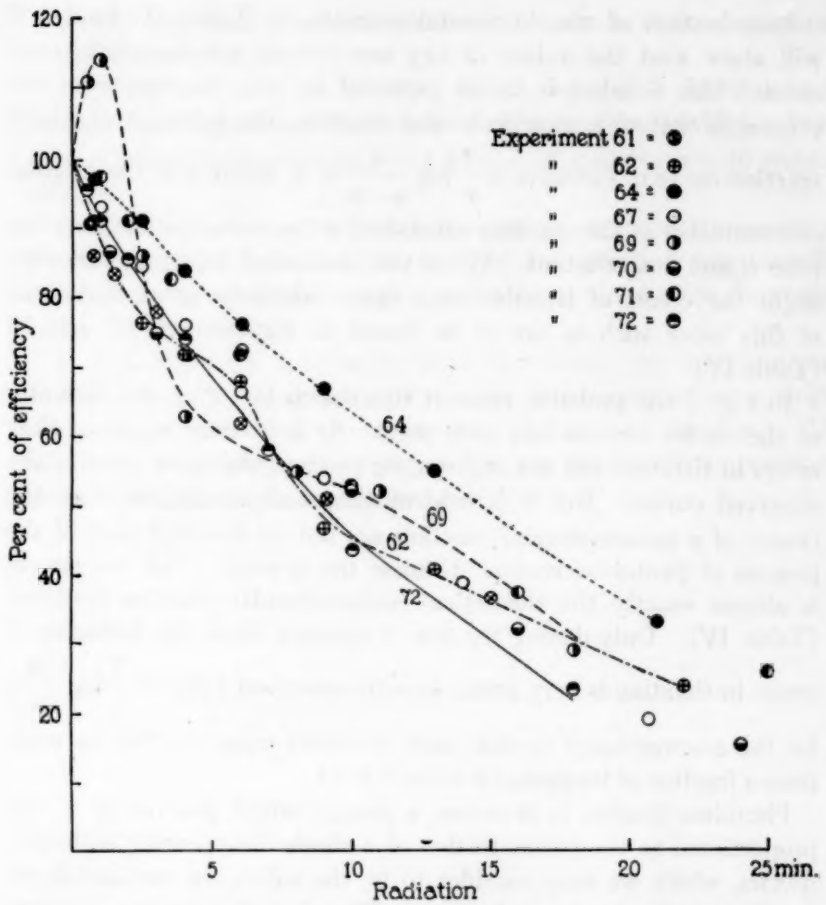

FIG. 2. Courses of the process of photoinactivation of eight samples of complement. Curves are drawn to show four characteristic examples. The ordinates represent efficiency in per cent of that of unradiated complement, and the abscissæ represent time of exposure to light in minutes.

study of a larger number of sera, yet those here presented will serve to demonstrate two facts; first, that the primary photoreaction is monomolecular, and second, that upon this primary monomolecular reaction there are superimposed accessory processes which serve to increase or decrease the hemolytic power of the serum.

Examination of the horizontal columns in Table IV headed k will show that the values in any one column are essentially constant. This is what is to be expected in case the course of the process is that of a monomolecular reaction, the equation of whose reaction curve or isotherm is $\frac{1}{t} \log \frac{a}{a-x} = k$, where a is the original concentration of the reacting substance, x its concentration after the time t, and k a constant. When the amount of injury is still very slight the errors of titration may cause relatively great variations of this value such as are to be found in Experiments 62 and 70 (Table IV).

In Fig. 2 the probable error of titration is less than the diameter of the circles surrounding each point. It is at once apparent that errors in titration will not account for the irregularity of some of the observed curves. But it is evident that such deviations from the course of a monomolecular reaction are not an essential part of the process of photoinactivation, because the average of all the curves is almost exactly the theoretical monomolecular reaction isotherm (Table IV). Only during the first 2 minutes, when the influence of errors in titration is very great, does the observed value of $\frac{1}{t} \log \frac{a}{a-x}$ for the average curve deviate from its mean value, 0.0289, by more than a fraction of its probable error, 0.0013.

Photoinactivation is therefore a process which goes on at a rate proportional to the concentration of a single disappearing molecular species, which we may consider to be the substance responsible for the hemolytic property of serum. The deviation of the observed courses of photoinactivation from monomolecular curves also demands explanation, but may best be considered in connection with evidence which will be presented in a subsequent paper.

SUMMARY.

The photoinactivation of complement has been studied with a view to determining if possible how many kinds of molecules disappeared during the reaction. It was found that:

1. The apparent course of photoinactivation is that of a monomolecular reaction.

2. Diffusion is not the limiting factor responsible for this fact, because the temperature coefficient of diffusion is much higher than that of photoinactivation (Q_{10} = 1.22 to 1.28, and Q_{10} = 1.10 respectively).

3. There is no change in the transparency of serum solutions during photoinactivation, at least for light of the effective wave-length, which is in the ultra-violet region probably at about 2530 Ångström units.

It is pointed out that under these conditions only one interpretation is possible; namely, that during photoinactivation a single disappearing molecular species governs the rate of reaction. This substance must be primarily responsible for the hemolytic power of serum when it is used as complement.

THE MECHANISM OF COMPLEMENT ACTION.

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It has been shown in the preceding paper that the hemolytic action of serum is to be attributed primarily to a single substance which is destroyed under the influence of ultra-violet light by a monomolecular reaction. This fact immediately raises the question of the nature of the photosensitive substance, and leads the writer to make some suggestions as to what such a substance might be.[1]

I.

Certain experiments of the writer seem definitely to exclude the possibility that the serum proteins are primarily responsible for the power of serum to act as complement in specific serum hemolysis, for this power can be abolished by the action of light without producing the sensitization to heat which is characteristic of the effect of light on serum proteins. These experiments, which are summarized in Table I, show that radiated complement is not thereby made more susceptible to injury by heating.[2]

If the rates of heat inactivation of any individual serum before and after radiation are compared, it will be noted that the differences of efficiency are so small as to be attributable to errors in titration;

* The experiments upon which this paper is based were done by the writer as Research Fellow in the Harvard School of Tropical Medicine.

[1] In this paper the word "complement" will be used to denote any solution of serum used as one component in specific serum hemolysis (the other component being amboceptor); while that particular ingredient of serum primarily responsible for its hemolytic power is designated as the "lytic substance" or "lytic principle."

[2] All three experiments were done before the development of dependable methods of titrating complement, and a probable error of about ±3 per cent is to be expected.

and furthermore that the average value of the rate before radiation is almost exactly the same as the average value after radiation. This shows that radiation does not sensitize complement to heat. Experiments conducted for another purpose incidentally supply further proof that at temperatures up to 37°C. radiated complement deteriorates at the same rate as it did prior to radiation.[3]

Now Bovie[4] and Chalupecky[5] independently pointed out that ultraviolet radiation sensitizes such proteins as egg white and lens protein

TABLE I.

Summary of Experiments on the Effect of Heating upon Radiated and Normal Complement. Velocities Calculated as $k = \frac{1}{t} \log \frac{a}{a-x}$.

Experiment No.	Radiation.		Heating.					Ratio of velocities, Radiated: Normal.
	Exposure.	Injury.	Sample.	Bath temperature.	Exposure.	Injury.	Velocity.	
	min.	*per cent*		°C.	*min.*	*per cent*		
17	2	24	Radiated.	55.5	3	23.5	0.1165	1.25
			Normal.	55.5	3	19.3	0.0935	
34	45	87	Radiated.	52.0	11	84.0	0.77	1.23
			Normal.	52.0	11	76.0	0.624	
42	15	44	Radiated.	45.2	6	5.0	0.0056	0.55
			Normal.	45.2	6	9.0	0.0102	
Average								1.01

to subsequent heating, so that they coagulate much more rapidly or at lower temperatures. In Bovie's experiments radiated egg white coagulated even at room temperature. Schanz[6] showed that ultraviolet light has a similar effect on serum proteins; he also confirmed and extended the findings of Chalupecky on egg white and lens proteins.

If the hemolytic power of the serum were due to its protein content, radiation sufficient to destroy a large part of that power should be

[3] Brooks, S. C., *J. Med. Research*, 1920, xli, 411.

[4] Bovie, W. T., *Science*, 1913, xxxvii, 373.

[5] Chalupecky, H., *Wien. med. Woch.*, 1913, lxiii, 1986.

[6] Schanz, F., *Arch. ges. Physiol.*, 1915, clxi, 384.

accompanied by changes which are at least qualitatively like those displayed by the pure proteins themselves under radiation. Since the lytic principle can be nearly destroyed without any perceptible alteration of the serum proteins, we are forced to the conclusion that complement is not one of the serum proteins, and is, to a certain extent at least, independent of them.

II.

This must not be thought to mean that the hemolytic power of serum is wholly independent of proteins under all conditions. The experiments of Jacoby and Schütze[7] on the inactivation of complement by shaking, those of Michaelis and Skwirsky[8] on the effect of proteases on complement, and many others indicate the contrary.

To obviate confusion it may be well to point out that while the hemolytic substance is probably not a protein, as explained in the preceding section, this is not incompatible with the fact that complement may be profoundly affected by changes in the serum proteins; inactivation may be the direct effect of some agent, *e.g.* light, or it may be indirect and due to the effect of a change produced by the action upon serum proteins of such an agent as a protease. The serum proteins thus altered may then act upon the lytic substance.

The following experiments bear out this idea, since they suggest that acid may inactivate complement not by means of its effect upon the lytic substance itself, but by altering the state of the serum proteins.

It has long been known that complement could be made inactive by the addition of acid and alkali, and that if more than a small amount of acid or alkali was added the inactivation was not reversible by subsequent neutralization of the added reagent. The precise limits of pH value between which inactivation is still reversible have not heretofore been determined.

Fresh guinea pig serum was diluted to 20 volumes with 0.85 per cent NaCl solution[9] and kept in containers immersed in ice water

[7] Jacoby, M., and Schütze, A., *Berl. klin. Woch.*, 1909, xlvi, 2139.

[8] Michaelis, L., and Skwirsky, P., *Z. Immunitätsforsch., Orig.*, 1910, vii, 497.

[9] The balanced solution employed for dilution throughout all other parts of this work would have resisted changes in reaction by reason of its bicarbonate content, and hence could not be used in this particular operation.

at all times except for one period of about ½ hour while the acid was acting; during this period a temperature of 10°C. was maintained. Three solutions were prepared for admixture with the complement: 0.04 N HCl, 0.04 N NaOH (adjusted by comparison with HCl), and a 0.02 N NaCl solution made by mixing equal volumes of these two solutions. All these solutions were cooled to the temperature of the diluted complement before mixing.

The general plan of the experiments involved the addition of the desired amount of acid, and after a certain length of time its neutralization by the equivalent amount of alkali. In each case enough 0.02 N NaCl was added to make the final volume and concentration the same in all the tubes. The effect of the change in NaCl concentration (from 0.85 to 0.73 per cent) was negligible in view of the subsequent dilution with much larger amounts of balanced solution. The dilution of the serum was taken into account in the calculations.

Sets of clean sterile test-tubes were set in an ice water bath, each set consisting of a tube in which was placed 10 cc. of 5 per cent complement, and tubes for the acid, alkali, and NaCl solutions. At the beginning of the experiment the samples of complement were in turn poured into the tubes containing acid and quickly mixed by pouring back and forth twice. After the desired interval these mixed samples were poured in like manner into the tubes containing alkali, and then into the final tube containing NaCl to equalize the volumes. The same operations were performed even when no acid or alkali was to be added, to obviate any possible effect due to the slight foaming involved in mixing the samples even though this caused no demonstrable inactivation.

After this treatment the complement was further diluted to the desired extent and titrated as described in a previous paper,[10] by a method in which the probable error of determining the relative efficiency of samples of complement lies between 1 and 2 per cent.

The action of the acid apparently takes place very quickly; certainly in less than 15 minutes at 0°C. A preliminary experiment will serve to demonstrate this point. The efficiencies of two samples of complement left in the acid for 15 and for 66 minutes respectively

[10] Brooks, S. C., *J. Med. Research*, 1920, xli, 399.

were 88.6 and 89.7 per cent respectively. These figures differ by considerably less than the probable error and are therefore to be considered identical. In the remaining experiments the acid was allowed to act for about 30 minutes (the time varying by not more than ½ minute in any one experiment) so that there could be no doubt that it would have its full effect.

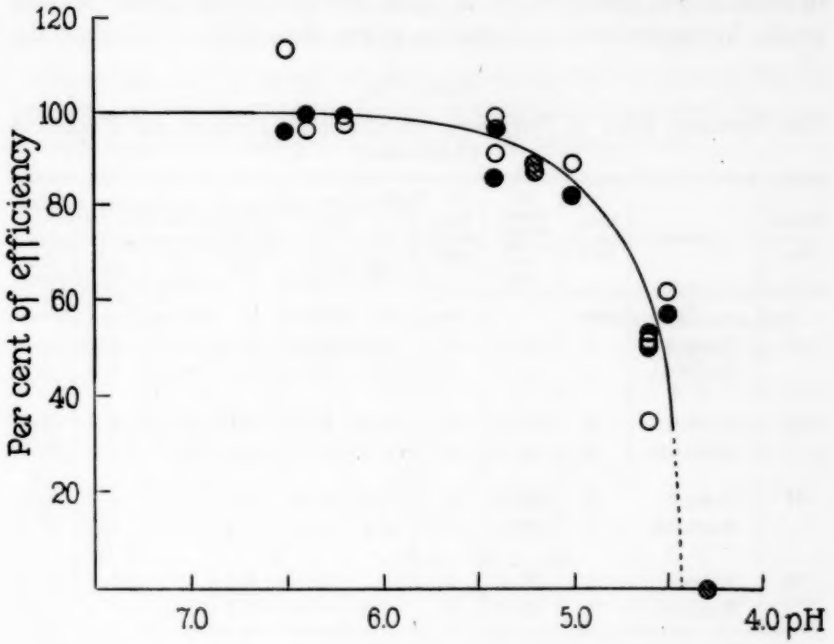


FIG. 1. The effect of temporary acidulation upon the subsequent efficiency of radiated and normal complement. The ordinates represent efficiency in per cent of that of the corresponding unacidulated complement, and the abscissæ represent the pH to which the complement was exposed during the period of acidulation. Open circles, normal complement; solid circles, radiated complement; where these coincide the circles are shaded.

The relation between the true reaction during acid treatment and the hemolytic activity after the restoration of the normal reaction of serum (pH = 7.6) will be most easily grasped by a study of Fig. 1 in which relative efficiencies are plotted as ordinates against hydrogen ion concentration in terms of pH. These values of pH are accurate

only within 0.2 pH units, because they were judged only on the basis of the approximate colors, in samples of the acidulated complement, of two or more of the following indicators; phenol red, brom cresol purple, methyl red, and brom phenol blue. No standard comparison solutions were used. Bearing these facts in mind it will be seen that the experiments agree in showing very little change in the efficiency of complement (perhaps even a slight increase in some cases) as long as the hydrogen ion concentration is less than pH = 6.0; when the

TABLE II.

The Permanent Effect of Temporary Acidulation on Radiated and Unradiated Complement.

Experiment No.	Sample.	Exposure to light.	Efficiency normal = 100 per cent.	Exposure to acid.	Efficiency at indicated pH; referred to corresponding unacidulated sample as 100 per cent.					
					pH	Efficiency.	pH	Efficiency.	pH	Efficiency.
		min.		*min.*		*per cent*		*per cent*		*per cent*
90	Normal.	0	100.0	33	6.5	113.1	5.4	91.0	4.3	0.0
	Radiated.	4	76.3	32	6.5	95.8	5.4	85.8	4.3	0.0
96	Normal.	0	100.0	31	5.4	99.2	5.0	87.2	4.6	33.8
	Radiated.	5	92.7	31	5.4	95.5	5.0	82.4	4.6	53.0
97	Normal.	0	100.0	31	6.4	96.3	5.2	86.9	4.6	50.7
	Radiated.	5	73.1	31	6.4	99.3	5.2	87.1	4.6	50.5
98	Normal.	0	100.0	35	6.2	96.8	5.2	88.8	4.5	61.6
	Radiated.	6	86.7	35	6.2	98.6	5.2	88.8	4.5	56.6

pH = 5.5, there is some decrease in the hemolytic power, and as it exceeds 5.0 there is a rapid drop in efficiency, complete inactivation being the result at pH = 4.3. The data for these experiments are given in detail in Table II.

There is a remarkable parallelism between this behavior and the known characteristics of the serum proteins. The serum proteins are usually classified as eu- and pseudoglobulins and albumins according to their solubility in water and in salt solutions; but there are no sharp divisions between these groups. The isoelectric point of euglobulins as prepared by Rona and Michaelis[11] is given by them as 3.6

[11] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.

$\times 10^{-6}$, which is equivalent to a pH of about 5.4; that is, the point at which complement begins to be affected by acid. The hydrogen ion concentration at which the inactivation of complement becomes complete is about pH = 5.0; this is just on the acid side of the isoelectric point of the serum albumin, which is at pH 4.7.[12]

Now amphoteric substances, among which we must number the serum proteins, behave as anions on the alkaline side of their isoelectric points, and as cations on the acid side of their isoelectric points; while at this point the sign of their charge changes from negative to positive, and there are great changes in their physical properties corresponding to slight changes of hydrogen ion concentration.[13]

TABLE III.

The pH of Samples of the Same Lot of 5 Per Cent Complement Radiated for Different Lengths of Time.

Exposure.	Efficiency.	Hydrogen ion concentration.
min.	*per cent*	pH
0	100.0	7.55
½	95.7	7.52
1	90.9	7.52
2	85.6	7.52
4	74.0	7.55
7	57.6	7.50
10	43.6	7.55
18	23.6	7.55

Since the hydrogen ion concentration at which these changes occur is coincident with that at which complement loses its hemolytic power, it is not improbable that there is a connection between the phenomena. We might suppose that the protein cation takes part in an irreversible reaction with the lytic substance or some one of its constituent groups. Other possibilities might be suggested, but the evidence is not adequate for distinguishing between one possibility and another, and the essential point remains the same; that there is some close connection between the state of the serum proteins and the effectiveness of the lytic substance.

[12] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1911, xxxiii, 456.

[13] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

If this is true then the lytic substance may be inactivated in either of two ways: either directly, as by ultra-violet light; or indirectly by means of agents affecting other serum constituents; *e.g.*, the proteins[14] (Table III).

These same experiments show also that radiation sufficient to destroy the hemolytic power of serum may fail to have any appreciable effect on the serum proteins; otherwise there should be a difference between radiated and normal complement in their susceptibility to inactivation by acids (Fig. 1 and Table II). Differences between radiated and normal complement do occur occasionally, but they are so irregular as to have no obvious general significance. Radiation fails to sensitize complement to either heating (Section I) or acidulation, and the two sets of experiments substantiate each other in this respect.

III.

In view of the fact that surface tension of complement has been thought by some[15, 16] to be connected directly with its hemolytic power it is interesting to note the change in surface tension taking place when complement is inactivated by ultra-violet light.

The surface tension of 5 per cent complement solution was measured by means of a Traube stalagmometer and was found to decrease slightly (Table IV and Fig. 2). Measurements of the time of outflow made at the same time are probably not reliable because of the presence of occasional wisps of cotton in the solutions, but since they seem to indicate a change in viscosity they are also given: if there is any definite change of viscosity it is a decrease.

[14] In this connection it should be pointed out that changes in hydrogen ion concentration play no part in the ordinary course of photoinactivation. Samples of 5 per cent complement were taken immediately after radiation for various lengths of exposure and their hydrogen ion concentration determined by the addition of an appropriate amount of phenol red and comparison of the resulting color with that produced in solutions of known hydrogen ion concentration. The least active sample had been reduced to a relative efficiency of 23.6 per cent. There was an irregular variation of between pH = 7.55 and 7.50, which is so small as to be utterly negligible (Table III). The complement is at all times exposed to a hydrogen ion concentration the same as that prevailing in the blood plasma.

[15] Traube, J., *Biochem. Z.*, 1908, x, 371.

[16] Traube, J., *Biochem. Z.*, 1908, x, 380.

TABLE IV.

The Surface Tension and Viscosity of 5 Per Cent Complement Solutions Radiated for Different Lengths of Time.

Experiment No.	Exposure.	Efficiency.	Outflow from stalagmometer.		
			No. of drops.	Time of outflow	
	min.	*per cent*		*min.*	*sec.*
71	0	100.0	56.4	5	17
	2	91.0	56.4	5	5
	3½	82.4	57.7	5	13
	10	52.4	57.8	4	45
	Diluent alone.		56.0	4	53
72	0	100.0	56.3	5	2
	½	95.7	57.1	4	59
	2	85.6	57.2	4	54
	8½	50.0	57.25	4	46
	Diluent alone.		56.1	5	5

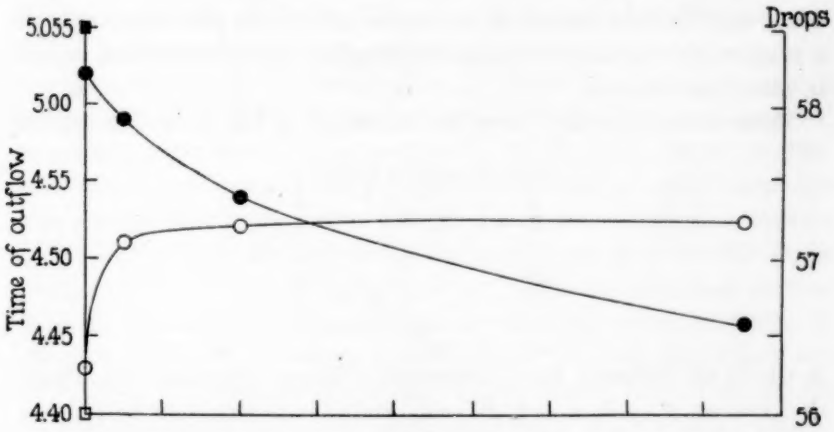


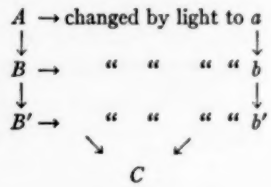
FIG. 2. The progressive changes in surface tension and viscosity of complement during photoinactivation. The squares indicate the data for the diluent alone. The ordinates represent the time, or the number of drops required for the outflow of a constant amount of complement from a Traube stalagmometer. The abscissæ represent in minutes the time of exposure of the samples to the light. Open circles and square, surface tension; solid circles and square, viscosity.

The change of surface tension is like that accompanying thermo-inactivation,[17] and contrary to the hypothesis advanced by Traube,[15, 16] is probably not the cause of the change in hemolytic power.[17]

IV.

In the following paragraphs there is suggested an hypothesis which has the advantage of explaining many of the known properties of serum complement with more definiteness than current theories and without being in conflict with any well established fact. Definite substances are named only for the sake of making it easier to grasp certain essential ideas, and not with any pretense that complement must be supposed to consist of just these particular substances. The main ideas are these: that there is a hemolytic substance in serum which is constantly breaking down into non-lytic material, and constantly renewed from a store of some "precursor" substance; that this lytic substance is so related to the serum proteins that it is more or less permanently inactivated by certain changes in the serum proteins; and that the hemolytic substance and all its precursors contain a certain photosensitive molecular grouping whose alteration results in photoinactivation.

These essential points may be embodied in the following scheme



in which the different letters represent different chemical individuals; the changes $A \rightarrow B$, $a \rightarrow b$, $B' \rightarrow C$, and $b' \rightarrow C$ represent hydrolyses, and the changes $B \rightarrow B'$ and $b \rightarrow b'$ the passage of B or b from solution in fats to solution in water as a result of a change in relative solubility produced by the preceding hydrolysis. B (*i.e.* $'B'$) is the principal lysin, and b', formed by radiation, is probably also hemolytic to a certain extent.

When complement is heated we need consider only the left-hand series, $A \rightarrow B \rightarrow B' \rightarrow C$. A is a precursor present in large amount

[17] Schmidt, H., *J. Hyg.*, 1913, xiii, 314.

in serum, and undergoing transformation into B at a rate which is not very greatly accelerated by an increase in temperature. The change $B \rightarrow B'$ is very rapid and is probably never a limiting factor. B' is the lysin and undergoes hydrolysis at a rate markedly influenced by changes of temperature, and proportional to the amount of B' present at the moment, as in any monomolecular reaction such as most hydrolyses.

Let us suppose serum to be collected and placed at 0°C. The breakdown of B' may be retarded by the lowered temperature, and B' will accumulate until there is so much that the amount broken down in a unit of time will equal the amount formed from A. (We may suppose the process $B \rightarrow B'$ to be so rapid as to have no perceptible influence on variations in the amount of B'.) Since the reaction $A \rightarrow B$ is also monomolecular, the amount of B formed in any time interval will be less and less as A is used up; and to keep pace with this change B', the lysin, must also gradually decrease in amount. Thus the hemolytic power, which is proportional to the amount of B', will gradually decrease. This is a well known phenomenon.

Let us suppose that serum which has reached this steady state of equal formation and destruction of B' is suddenly raised to a high temperature, *e.g.* 56°C.; the destruction of B', which we have assumed to have a high temperature coefficient, will be enormously accelerated while its formation will still be relatively slow. As a result the hemolytic power of the serum will rapidly decrease, and, since its rate of formation is at first relatively negligible, the decrease will follow the course of a monomolecular reaction. This will continue until so little B' is left that it decomposes at a rate comparable with that of the change $A \rightarrow B$. Madsen and Watabiki[18] present data which not only show that thermoinactivation of complement follows the course of a monomolecular reaction (about as closely as photoinactivation) but that the temperature coefficient at 50–56°C. is very high (Q_{10} lying between 123 and 366.8), but between 3 and 37°C. is about that of typical dark reaction (Q_{10} between 1.98 and 2.94).[19]

[18] Madsen, T., and Watabiki, T., *Oversigt. kong. danske Videnskab. Selskabs Forhandl.*, 1915, 125.

[19] Madsen and Watabiki express their results in terms of the temperature coefficient of the van't Hoff-Arrhenius formula. The values of Q_{10} given above are calculated directly from their data.

This agrees exactly with our hypothesis, both as to the order of the reaction of thermoinactivation, and in that the destruction of B' with its high temperature coefficient is predominant at temperatures above 50°, while the limiting reaction at temperatures below 37° is the breakdown of A which has a relatively low temperature coefficient. The gradual decrease of B' then keeps pace with the gradual decrease of A. Whenever, as after brief heating to 56° for example, the concentration of B' is disproportionately reduced and a large amount of A is still left, the latter will act as a reservoir from which B' will be restored to the concentration appropriate to the temperature and the remaining amount of A. The hemolytic power being proportional to B' will be low immediately after heating and will then be regenerated as Gramenitzki[20] and the writer[1] have shown to be the case.

Turning now to the question of what occurs during photoinactivation we find that if the time curves of several experiments are averaged the process follows the course of a monomolecular reaction.[21] Now if only B' were hemolytic and the members of the left-hand branch of our hypothetical series (*i.e.* A, B, and B') contained the same photosensitive group, the proportion of each substance destroyed would be the same; that is, one-tenth or one-half or nine-tenths of each of them would be destroyed, but not one-half of A and nine-tenths of B and B'. Under certain conditions which were defined in a previous paper[21] the destruction of a substance by light will follow the course of a monomolecular reaction, and if B' is so destroyed, and no disproportionate amount of A and B is left (the latter case occurs when complement is briefly heated), then there can be no regeneration. The theory then accounts for the observed course of photoinactivation and for the fact that no regeneration follows photoinactivation.

The reader should now note carefully the curves shown in Fig. 2, of the preceding paper. They are all characterized by the fact that at some time during the process the hemolytic activity exceeds that to be expected if the process followed exactly the course of a monomolecular reaction; there is a "wave" of excess activity. If we suppose that b' is hemolytic and that it forms and decomposes at a relatively

[20] Gramenitzki, M., *Biochem. Z.*, 1912, xxxviii, 501.

[21] Brooks, S. C., *J. Gen. Physiol.*, 1920–21, iii, 169.

rapid rate it is easy to see that as A is changed to a the concentration of a will increase; later as A is used up a will decrease again. Similarly b and b' will appear, increase, and disappear. If b' is hemolytic there will be a "wave" of hemolytic power in addition to that due to B' and not only the average time curve of photoinactivation, but also the divergence of individual curves from this average is satisfactorily accounted for. The time at which this wave occurs in the course of the process will depend on the rate of the slower of the processes, a to b and b to b'; presumably this is a to b which may well be dependent on an enzyme whose concentration is different in different samples of serum. If the conditions are favorable we may expect the wave of excess hemolytic power to be so large and so early that the complement becomes more efficient when radiated, as during the early part of Experiment 69 of the preceding paper.[22]

In order to render this hypothesis more tangible it is desirable to suggest the nature of the substances A, B, a, b, and so on, even if there is no direct evidence for their exact composition. There is considerable evidence that fatty acids are important in immune reactions: Warden[23] has synthesized from fatty acids antigens which produce specific antibodies against blood cells and gonococcus; Jobling and Bull[24] have shown that the lipase content and hemolytic power of human sera vary together. Noguchi,[25] among others, has shown striking analogies between complement and solutions of fatty acid compounds dissolved in serum albumin solutions. All these facts suggest the presence of a fatty acid compound acting as hemolysin in complement.

The most active hemolysin of which the writer has found any record is the "lysocithin" produced by the action of cobra venom upon crude lecithin and studied by Fourneau and his students. It appears to be at least 50 times as active as saponin and perhaps 100 times as active as the most hemolytic soaps. It is a fatty acid compound: choline monopalmitoglycerophosphate.[26] It should be noted that

[22] Brooks, S. C., *J. Gen. Physiol.*, 1920–21, iii, 180, Table IV; and 181, upper curve of Fig. 2.

[23] Warden, C. C., *J. Infect. Dis.*, 1918, xxiii, 504.

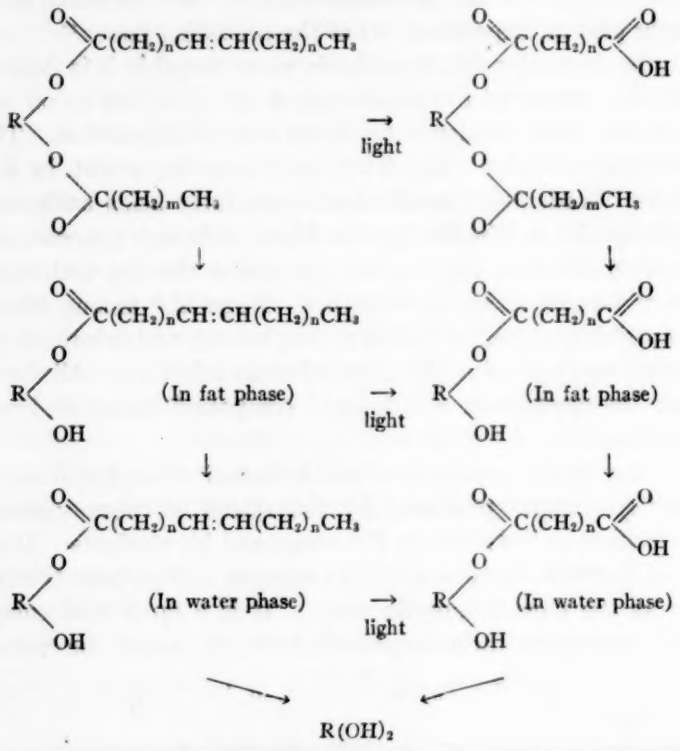
[24] Jobling, J. W., and Bull, C. G., *J. Exp. Med.*, 1913, xviii, 61.

[25] Noguchi, H., *Biochem. Z.*, 1907, vi, 327.

[26] Fourneau, E., and Delezenne, C., *Bull. Soc. Chim.*, 1914, series 4, xv, 354.

this substance is soluble in warm water, and hardly soluble in benzene, while complement is an aqueous solution, and is not easily injured by extraction with benzene.[27] Lysocithin also forms an irreversible compound with emulsified cholesterol, thus reminding one of the fact that emulsions of cholesterol "fix" complement. Furthermore, lysocithin is formed by the action of venom lipase on lecithin but is destroyed by the further action of the same agent; removal of the second fatty acid leaves the lecithin complex inactive.[28] These two steps might be compared with the process which results in spontaneous deterioration of complement.

With these facts in mind we may proceed to picture the nature of the hemolysin system in complement as follows:



[27] Schmidt, P., *Z. Chem. Ind. Koll.*, 1912, xi, 5.

[28] Delezenne, C., and Fourneau, E., *Bull. Soc. Chim.*, 1914, series 4, xv, 421.

In this scheme R represents choline glycerophosphoric acid or some similar substance, and, since compounds of lower fatty acids are markedly lacking in hemolytic power, the two fatty acids may each be supposed to contain at least ten carbon atoms. One of the acids is for two reasons supposed to be unsaturated: because compounds of unsaturated fatty acids are in general more hemolytic than those of the corresponding saturated acids, as shown by Lamar;[29] and because this furnishes a molecular grouping known to be attacked by light[30] with a consequent break in the fatty acid chain which might be expected to bring about a great decrease in hemolytic power.[31]

Many phenomena displayed by complement are undoubtedly dependent in some way upon the physical or chemical state of the serum colloids, as is shown for example in the previously noted inactivation by acid. Sachs and Stilling[32] have shown that inulin suspended in cold water affects complement, while if first dissolved by warming the water it no longer has any effect. It is not surprising then that complement is inactivated by shaking, since shaking, or rather the attendant foaming, causes irreversible coagulation of proteins,[33] nor that processes which precipitate serum globulins should, if the process is reversible, produce the so called "fractions" which upon being recombined regain their hemolytic power. The conflicting nature of the evidence about these so called fractions, as well as the writer's own experience with several methods of obtaining the fractions, leads him to doubt the uniformity of the preparations secured by different investigators. Evidently the lytic substance is usually held inactive in one of the fractions, since exposure of sensitized red blood cells to the globulin or "mid-piece" fraction results in a change which makes them susceptible of lysis by the albumin fraction, which is therefore supposed to combine with some element in the red blood cells indirectly through the "mid-piece." It is for this reason that the albumin fraction has received the name "end-piece."

[29] Lamar, R. V., *J. Exp. Med.*, 1911, xiii, 380.
[30] Ciamician, G., and Silber, P., *Ber. chem. Ges.*, 1914, xlvii, 640.
[31] Shimazono, J., *Arch. exp. Path. u. Pharmakol.*, 1911, lxv, 361.
[32] Sachs, H., and Stilling, E., *Z. Immunitätsforsch., Orig.*, 1917, xxvi, 530.
[33] Ramsden, W., *Arch. Physiol.*, 1894, 517.

When cobra venom is allowed to act upon complement under certain conditions there ensues a change in the serum such that when this cobra serum, as it is called, is combined with either mid- or end-piece of normal complement, or when either fraction of the cobra serum is combined with the supplementary fraction of normal serum the mixture is hemolytic.[34] This is supposed to be due to the action of cobra venom upon a "third component" of complement. Hemolysis by complement would then be considered to result from the combined action of mid-piece, end-piece, and third component, all of which must be present.

The writer is inclined to regard the cobra serum as serum from which all the lysin and its precursors have been removed. The third component, which is destroyed by cobra venom, is the lysin and its related substances. This is in accord with the work of Delezenne and Fourneau[28] and with the observed characteristics of cobra serum.

Phenomena susceptible of explanation in terms of the scheme here proposed abound in the literature, and their descriptions might be multiplied indefinitely if anything were to be gained by so doing. It is of greater significance that search of the voluminous literature on complement has so far failed to discover any well established fact which is incompatible with this hypothesis. If such facts are found, the hypothesis must of course be replaced by some better one; but the writer believes that the main ideas underlying it, namely successive transformations of precursor into lysin and then into inactive products, and the dependence of this lysin on the serum proteins, will prove to be valid. At any rate they seem preferable to certain prevailing explanations based upon the rather indefinite concept of a hemolytic power resulting from the colloidal properties or "lability" of serum proteins.[35]

SUMMARY.

It has been shown:

1. That complement exposed to ultra-violet light is not thereby sensitized to the action of heat (which indicates that it is not protein).

[34] Ritz, H., *Z. Immunitätsforsch., Orig.*, 1912, xiii, 62.

[35] Such ideas have been expressed by many investigators and have recently been developed and emphasized by Sachs in a review of the work of his laboratory (Sachs, H., *Koll. Z.*, 1919, xxiv, 113).

2. That inactivation of complement by ultra-violet light is accompanied by a decrease in its surface tension.

3. That photoinactivation of complement is not a result of any changes in hydrogen ion concentration since these are less than 0.05 pH.

4. That hydrogen ion concentrations high enough to transform serum proteins from the cation to the anion condition (*i.e.* past the isoelectric point) permanently inactivate complement.

These facts together with those given in previous papers lead to the following hypotheses.

1. That there is present in serum a hemolytic substance which is formed from a precursor (which may resemble lecithin) and is constantly being formed and simultaneously being broken down into inactive products.

2. That both precursor and lysin contain the same photosensitive molecular group.

3. That the lytic substance is dependent for its activity upon the state of the serum proteins.

COMPARATIVE STUDIES ON RESPIRATION.

XIII. An Apparatus for Measuring the Production of Minute Quantities of Carbon Dioxide by Organisms.

By MARIAN IRWIN.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, June 28, 1920.)

Osterhout[1] has described an apparatus by means of which the production of small quantities of carbon dioxide by organisms may be measured at short intervals. In this apparatus air is caused to circulate through a tube containing the organisms and then into a tube containing an indicator, the color changes of which are compared with buffer solutions containing the same amount of indicator.

The purpose of the present article is to describe a modified apparatus devised by the writer.

The whole apparatus, with the exception of a short piece of rubber tubing, is made of glass. In order to prevent leakage the joints and glass stop-cocks are ground with special care.

Fig. 1 shows a sectional view of a portion of the apparatus. The tube O contains the organisms and the tube I contains an indicator. These tubes are connected to the apparatus by ground joints. When the stop-cock S is opened, and the stop-cocks S_1 and S_2 are closed,[2] the air is allowed to pass from the tube O containing the organisms to the tube I containing the indicator.[3] When S is closed and S_1 and S_2 are opened, the air passes from O to I through N which contains lumps of sodium hydroxide for the absorption of carbon dioxide. The ground joint J allows the apparatus to be disconnected.[4] The dotted

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

[2] A single three-way stop-cock may be used in place of S, S_1 and S_2 in order to avoid dead spaces.

[3] The indicator tube and the inlet tube within it are made of Pyrex glass.

[4] It may be desirable to make the entire apparatus of Pyrex glass in which case the ground joint J may be transferred to the right of S. This facilitates the substitution of tubes of various sizes in place of O.

lines at X and Y indicate two glass tubes running off at right angles and connecting (at the points marked X_1 and Y_1 in Fig. 1, *B*) with another tube, composed partly of glass and partly of rubber. The central portion of this tube (R, Fig. 1, *B*) consists of a piece of rubber tubing[5] 2 mm. in thickness and 7 mm. in diameter (inside) which is joined to glass tubes at W and Z. The ends of the glass tubes are about 1 inch apart. In order to avoid leakage, the rubber tubing is

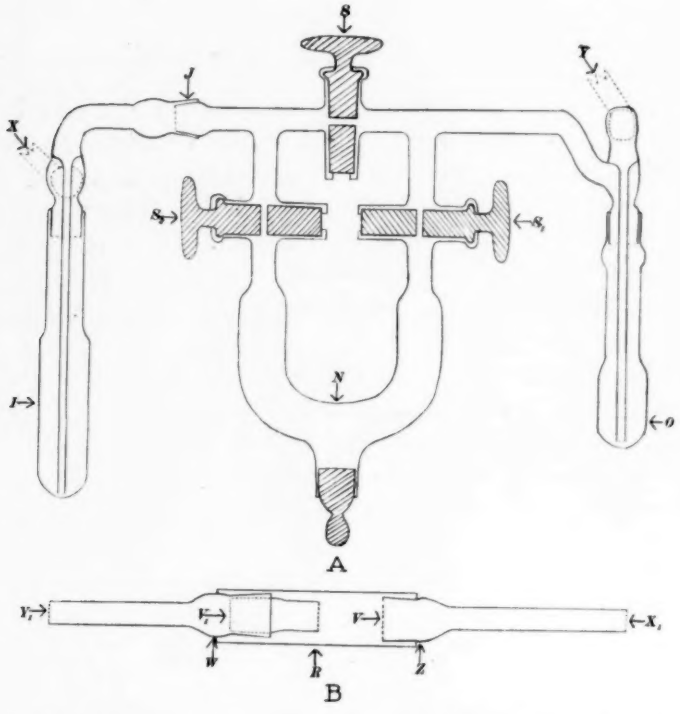


FIG. 1. (*A*) Apparatus for measuring the production of CO_2. Organisms are placed in the tube O and the CO_2 is carried over into the tube I which contains an indicator. The tube N contains NaOH for absorbing CO_2 when this becomes necessary.

(*B*) The rubber tube R is compressed at intervals, forcing air through the valves V and V_1 and so maintaining a circulation throughout the apparatus.

[5] Experiments are in progress with a view to replacing the rubber by other materials.

slipped over the glass for about 1 inch and cemented to the glass by soft de Khotinsky cement. V and V_1 show the position of the valves, which allow the air to pass in one direction only (from Z to W). The valves are made of thin rubber sheeting attached to perforated rubber stoppers.

In order to produce a circulation of air the piece of rubber tubing is alternately compressed and released by means of the device shown in Fig. 2. The motor operates a cam the turning of which causes the

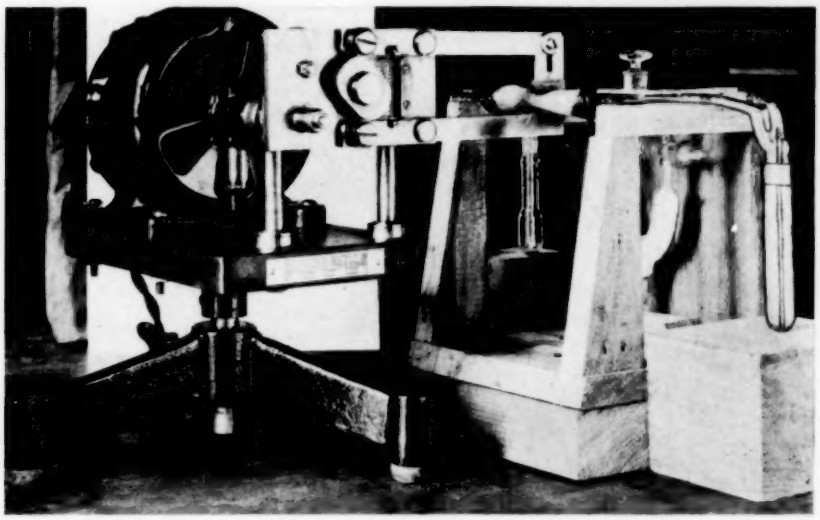


FIG. 2. The motor turns a cam which causes the horizontal arms alternately to approach and separate, thus compressing a piece of rubber tubing and maintaining a circulation of air in the apparatus.

two horizontal arms alternately to separate and to approach each other (they are held together by a vertical spring at the right of the cam[6]). Two projections at the ends of these arms are adjusted so as to compress the rubber tubing to the proper degree. The arms are made so that the height and the angle can be adjusted. The writer prefers a speed of 120 compressions per minute (the speed of the motor is reduced by gears).

[6] A worm may be substituted for the cam if noiseless operation is desired.

The glass portion of the apparatus is rigidly fastened to the wooden frame by two screws. The wooden frame, however, is not fastened to the table but is allowed to move freely to compensate for the slight motion produced by the compression of the rubber tube; without this freedom of motion the connecting glass tubes might be broken.

When a constant temperature must be maintained it may be desirable to immerse the apparatus in water. The motor must then be adjusted so that the arms which compress the rubber tubing occupy a vertical position. The water should be contained in a suitable glass vessel so arranged that the changes in the color of the indicator may be readily observed. The buffer solutions must also be immersed and should be placed beside the tube I which contains the indicator.

ENVIRONMENTAL FACTORS OTHER THAN TEMPERATURE AFFECTING FACET NUMBER IN THE BAR-EYED MUTANT OF DROSOPHILA.*

By JOSEPH KRAFKA, Jr.

(*From the Zoological Laboratory of the University of Illinois, Urbana.*)

(Received for publication, August 5, 1920.)

In a recent paper the author[1] has undertaken to show the relation between temperature and the facet number in the bar-eyed mutant of *Drosophila melanogaster* Meig. Since it was essential to know the part played by other environmental factors the experimental evaluation of some of these was carried out. While these experiments were not as critical as was desired, they give some idea as to the respective value of food, humidity, and evaporation as these are commonly represented in *Drosophila* culture technique. The following data are submitted, then, not as a final analysis, but rather as a basis for further work.

The work in all cases was carried out on the Ultra-bar mutant. For a direct comparison the data on the effect of temperature are given in Table I. The temperature data are for the interval 23–29°C., as the experiments involving the other factors were carried out at these temperatures.

An interesting suggestion for further work appeared in the analysis of the few experiments dealing with different kinds of foods. Since consistent results were obtained in three successive experiments it may be that they have some significance.

It is the experience of nearly all investigators rearing *Drosophila*, that fermented banana rapidly becomes acid at the high temperatures and that oviposition is much retarded. In an attempt to eliminate

* Contribution from the Zoological Laboratory of the University of Illinois, No. 165.

[1] Krafka, J., Jr., The effect of temperature upon facet number in the bar-eyed mutant of *Drosophila*, *J. Gen. Physiol.*, 1919–20, ii, 409, 433, 445.

the acidity, I tried raising mass cultures on pure Fleischmann's yeast made into a paste and covered with cotton-wool. To my surprise the cultures hatched 1 day earlier than did the banana control and an examination of the facet counts showed an appreciable difference in

TABLE I.

Effect of Temperature upon Facet Number in the Ultra-Bar Mutant of Drosophila melanogaster.

Temperature at which flies developed.	Mean facet number. Females.	Difference in facets per °C.	Mean facet number. Males.	Difference in facets per °C.
°C.				
23	28.30±0.24		31.43±0.24	
		1.53		1.91
25	25.24±0.09		27.60±0.10	
		1.98		1.95
27	21.27±0.10		23.70±0.11	
		2.02		2.34
29	17.23±0.08		19.02±0.08	
Average.....		1.84		2.07

TABLE II.

Effect of Various Foods upon Facet Number in the Ultra-Bar Mutant of Drosophila melanogaster.

Food on which larvæ were reared.	Experiment 55. Temperature 23°C.		Experiment 61. Temperature 23°C.		Experiment 71. Temperature 27°C.	
	Females.	Males.	Females.	Males.	Females.	Males.
Fleischmann's yeast..	23.72±0.34	27.37±0.27	22.40±0.18	25.01±0.18	18.28±0.24	19.85±0.74
Yeast foam...	27.53±0.73	28.80±0.82	24.04±0.26	26.92±0.32		
Banana..	26.69±0.62	32.20±0.48	29.54±0.34	32.50±0.27	20.80±0.34	22.96±0.12

the mean. A repetition of the experiment at 23 and again at 27°C. gave consistent results. What the actual factor is that underlies this phenomenon is still uncertain. The data are given in Table II.

Humidity has been one of the factors to receive considerable attention in the past few years. Since the apparatus for humidity and

temperature control were at hand several experiments were planned to test the action of that factor on development. Culture bottles, fitted up in the ordinary manner, were inclosed in humidity cases, one in which the humidity remained practically constant at 35 per cent, while the other was set for 60 per cent. Little or no difference was noted in the rate of development, or in the facet count (Table III).

Direct evaporation was next tried. Ordinary culture bottles were fitted with cork stoppers. A piece of glass tubing, extending through the cork to the surface of the banana, admitted air from the humidity control pipes. Another short tube with a cotton plug permitted

TABLE III.

*Effect of Humidity and Evaporation on Facet Number in the Ultra-Bar Mutant of Drosophila melanogaster.**

Atmospheric conditions under which immature stages were passed.	Mean facet number.		Range in facet number.	
	Females.	Males.	Females.	Males.
Culture bottle in 35 per cent humid air	26.59±0.32	28.86±0.37	21 to 32	23 to 38
Culture bottle in 60 per cent humid air	28.32±0.34	30.14±0.35	22 to 35	24 to 35
Direct evaporation 35 per cent humid air	Dried out. No larvæ.			
Direct evaporation 60 per cent humid air	32.21±0.71	34.48±0.90	16 to 47	26 to 49

* Temperature 23.5–25°C.

the escape of the air. The culture bottle, into which a stream of 35 per cent humid air was passed, dried up at the end of the 2nd day and no larvæ developed. The cultures into which 60 per cent air was admitted dried out rather rapidly, but not before many of the larvæ had pupated. Some of these emerged from pupation at the expected time but the majority was about 5 days late. The effect on the facet count is striking both as to the mean and the range. The upper range for the 23°C. stock counts is 39;[1] in this culture it has gone to 49, which is even above the upper range for 20°C. Unfortunately an atmometer test is impracticable, and no quantitative measure can be applied to this factor.

The foregoing experiments are of value in showing that when a consistent procedure is followed and plenty of good moist food is present, the environment is practically constant except for variations in temperature and need not be taken into consideration in the interpretation of the bulk of breeding data now available for *Drosophila*.

THE SIGNIFICANCE OF THE HYDROGEN ION CONCENTRATION FOR THE DIGESTION OF PROTEINS BY PEPSIN.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 25, 1920.)

One of the most striking peculiarities of enzyme action is the fact that the activity of the enzyme is limited to a definite range of acidity. If the solution is more or less acid than this the enzyme is practically inactive. Sörensen[1] showed that for a number of enzymes the determining factor was the hydrogen ion concentration and not the total acidity of the solution.

In attempting to account for this phenomenon it has usually been assumed that the influence of the hydrogen ion concentration was exerted upon the enzyme. Michaelis[2] pointed out, in the case of many enzymes, that if the activity of the enzyme was plotted against the hydrogen ion concentration of the solution the curve resembled strikingly that obtained when the ionization of a salt of a weak base and a strong acid was plotted in the same way. He concluded therefore that enzymes were weak bases or acids which formed salts with the acids or bases of the solution. These salts then dissociated into ions and the ions so formed were the active agents in the reaction. A similar explanation had already been proposed independently by Loeb[3] and by Nasse.[4] Michaelis' work rendered the hypothesis quite plausible. In the case of pepsin, however, it meets with several serious objections. In the first place, one of the strongest points of Michaelis' experiments was the fact that pepsin was found to have an isoelectric point at about pH 3.0 which agreed fairly well with the

[1] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.

[2] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 58.

[3] Loeb, J., *Biochem. Z.*, 1909, xix, 534.

[4] Nasse, O., *Malys Jahrb.*, 1894, xxiv, 718.

theory. Pekelharing and Ringer,[5] however, showed that in solutions of pure pepsin (prepared by Pekelharing's method from gastric juice) the pepsin was always negatively charged. This objection may of course be met by the statement that the pepsin under the actual conditions of hydrolysis (*i.e.* when in the protein solution) is not pure but is combined with some other substance and it is the ionization of this compound which determines the activity of the enzyme. An explanation similar to this has been offered by Michaelis.[6] The author[7] has shown, however, that pepsin combined with peptone or other decomposition products of the proteins is inactive and that it is only the free pepsin which takes part in the reaction. It was also found[8] that no positively charged pepsin could be found on the alkaline side of pH 3.3. Pepsin retains its activity up to pH 5, however, so that it seems unlikely that only positively charged pepsin is active, as assumed by Michaelis.

A second objection to Michaelis' view is the fact that the optimum hydrogen ion concentration for the activity of pepsin is found to vary with the substrate. This point has been emphasized by Long and Hull[9] (for trypsin) and by Ringer.[10] From Michaelis' point of view it is difficult to see how this can be. Neither of these objections, however, can in the author's opinion be considered as conclusive evidence against Michaelis' hypothesis. It could be stated for instance that pepsin contained several enzymes, one for each substrate and each with a different optimum. It seems simpler, however, to assume that the hydrogen ion concentration affects the condition of the substrate rather than the enzyme. This hypothesis has the advantage that it also accounts for the peculiar relation between the concen-

[5] Pekelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

[6] Michaelis, L., *Deutsch. med. Woch.*, 1920, xlvi, 1.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 468.

[9] Long, J. H., and Hull, M., *J. Am. Chem. Soc.*, 1917, xxxix, 1051. The same statement is made by Hedin and Hammerstein. The author has been unable to find the original work on which this statement is based. *Cf.* Hammerstein, O., and Hedin, S. G., A text-book of physiological chemistry, translated by Mandel, J. A., New York, 8th edition, 1915, 471. See also Abderhalden, E., and Fodor. A., *Fermentforsch.*, 1914–16, i, 591.

[10] Ringer, W. E., *Kolloid. Z.*, 1916, xix, 253.

tration of the substrate and the rate of hydrolysis. Experiments described in a former paper[11] show that the rate of hydrolysis of protein solutions of varying concentration but the same pH was directly proportional to the amount of ionized protein present in the solution but not to the total concentration of protein. They agree therefore with the hypothesis that the ionized protein is the form which takes part in the reaction. If this explanation is correct it follows that the optimum hydrogen ion concentration for the activity of pepsin is also due to the increased ionization of the protein and must coincide with the hydrogen ion concentration at which the protein solution contains the greatest number of protein ions. (It was first suggested by Pauli[12] that the ionized protein was the form which was attacked. Euler[13] and Arrhenius[14] have made a similar suggestion. Ringer[15] considers also that the ionization of the substrate has an influence on the rate of digestion at least in the later stages.) It should be possible therefore to determine the optimum degree of acidity for pepsin digestion by measuring the conductivity of the protein solution. It will be shown below that this is true. It will further be shown that the range of hydrogen ion concentration in which the enzyme is active shifts in the same sense as the conductivity of the protein solution when a protein of different isoelectric point is used, and also that when the protein is insoluble the enzyme combines with it only over that range of hydrogen ion concentration in which the enzyme is active and in which the protein is ionized.

The Influence of the Isoelectric Point of the Protein on the Activity of Pepsin at Different Hydrogen Ion Concentrations.

Ringer[10] has already shown that the optimum hydrogen ion concentration for the digestion of proteins by pepsin varies somewhat with the protein hydrolyzed and with the acid used. He accounts for this phenomenon by the assumption that the hydration of the

[11] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 595.
[12] Pauli, W., *Arch. ges. Physiol.*, 1910, cxxxvi, 483.
[13] Euler, H., Allgemeine Chemie der Enzymes, Wiesbaden, 1910.
[14] Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, 44.
[15] Ringer, W. E., *Arch. Neerl. Physiol.*, 1917–18, ii, 571.

protein determines the ease with which it is attacked by the enzyme. The viscosity is assumed to be a measure of the hydration. The same explanation has been proposed by Brücke,[16] by Pfliederer,[17] and recently by Traube.[18] The writer has been able to show,[19] however, that gelatin digests at the same rate in sulfuric or hydrochloric acid solution (provided the pH is the same) although the swelling, which Ringer considers a measure of the hydration, is much greater in hydrochloric than in sulfuric acid. It was also found[11] that the rate of digestion of egg albumin solutions decreased as the viscosity increased with the age of the solution instead of increasing as would be expected if the rate of digestion was determined by the hydration of the protein (as shown by the viscosity). Loeb[20] has shown that the ionization of the protein and the viscosity and swelling are all approximately proportional for a small range of acidity to the acid side of the isoelectric point. The maximum for the swelling and viscosity, however, occurs at about pH 3.4 whereas that for the ionization is much further to the acid side and agrees very well for that of the rate of digestion. This question will be discussed more fully below. It is clear, however, that in certain cases the swelling or viscosity and the ionization and rate of digestion may all be proportional. It would seem from the experiments described here that the determining factor for the rate of digestion is the ionization of the protein, and the swelling and viscosity are secondary characteristics which are probably also connected with the ionization.

It is known that, with most proteins, pepsin becomes inactive at a pH of about 4.5. This cannot be ascribed to the destruction of the enzyme since the author[8] found pepsin to be more stable in this range of acidity than at any other. The ionization of most proteins is very slight at this pH, however, so that it would be expected (from the hypothesis that it is the protein ion which is attacked by the enzyme) that little or no hydrolysis should occur at this point. Oxy-

[16] Brücke, E., *Sitzungsb. k. Akad. Wissensch. Math-naturw. Cl., Wien.*, 1859, xxxvii, 131.

[17] Pfliederer, R., *Arch. ges. Physiol.*, 1897, lxvi, 605.

[18] Traube, M., *Deutsch. med. Woch.*, 1919, xxvii,

[19] Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.

[20] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39; 1920–21, iii, 85.

hemoglobin, however, is isoelectric at a pH of about 6.8 (Michaelis[2]) so that it must be quite largely present as a salt and therefore ionized at a pH of 4.5. It would be predicted then, according to the hypothesis that the amount of protein ions present determines the rate of digestion of the protein, that hemoglobin should be digested by pepsin at pH 4.5 more rapidly than is egg albumin or gelatin at the same pH.

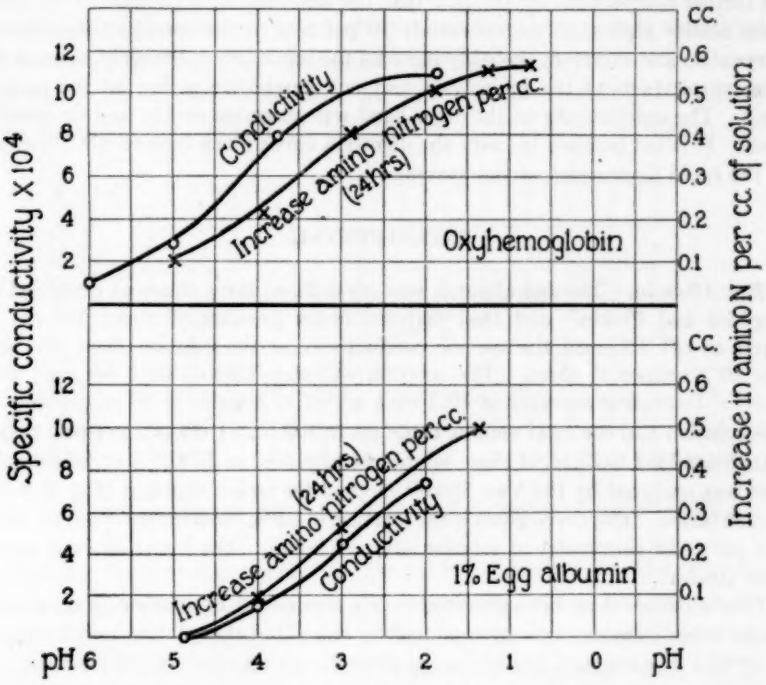


FIG. 1. Influence of pH on conductivity and rate of digestion of egg albumin and oxyhemoglobin solutions.

In order to test this prediction, parallel experiments were made to determine the rate of digestion and the conductivity of hemoglobin and egg albumin solutions at various hydrogen ion concentrations. The results of such an experiment are shown graphically in Fig. 1. It is clear that the conductivity and digestion curves, for each protein, as plotted against the pH of the solution are approximately parallel

and also that the curves for the digestion and conductivity of the hemoglobin fall further to the left (*i.e.* to the alkaline side) than do the curves for the egg albumin.

The experiments cannot be considered as showing quantitative agreement between the rate of digestion and the conductivity of the solution since the digestion curve is given as the amount of protein decomposed in a certain time—a quantity which is not connected in any simple way with the rate of digestion. They are further complicated by the fact that the digestion in the region of the optimum acidity represents approximately 50 per cent of the complete digestion of the protein and therefore probably includes the secondary splitting of some of the primary products of the hydrolysis, and not purely the action on the protein itself. The conductivity on the other hand was measured on the protein solution itself. It is not possible to carry the digestion curve much beyond pH 5.0 owing to the rapid destruction of the enzyme.

EXPERIMENTAL.

Egg Albumin.—The egg albumin was crystallized three times as described by Hopkins and Pinkus[21] and then dialyzed under pressure of about 150 cm. of water at pH 4.8 until the specific conductivity of the solution was less than 1×10^{-4} reciprocal ohms. The solution was then diluted to 2 per cent with water. Increasing amounts of HCl were added to a series of 50 cc. portions of this solution and the total volume made up to 100 cc. 1 cc. of 2 per cent pepsin was then added to 25 cc. of these solutions and placed at 25°C. 1 cc. of the solution was analyzed by the Van Slyke[22] method for amino nitrogen after 0, 8, 24, and 36 hours. The curve given is the increase in cubic centimeters of amino nitrogen per cubic centimeter of solution after 24 hours. The 8 and 36 hour curves were similar.

Conductivity.—1 cc. of inactivated pepsin was added to another 25 cc. portion of the above solutions and the conductivity and pH of the solution were measured at 25°C. The conductivity of the egg albumin salt was determined from the conductivity of the solution by subtracting from the observed conductivity the conductivity of HCl of the same pH (Northrop[11]).

Oxyhemoglobin.—Erythrocytes from fresh defibrinated ox blood were washed with 7.8 per cent glucose solution until the conductivity of the suspension was less than 1×10^{-4} reciprocal ohms. The cells were then laked with ether, separated from the excess ether, and the ether in the solution removed *in vacuo*. The solution was then diluted to contain about 1 cc. of amino nitrogen per cubic centimeter as determined by the Van Slyke method. The conductivity of this

[21] Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898–99, xxiii, 130.

[22] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

solution was about 1×10^{-5} reciprocal ohms. Increasing amounts of HCl were added to 50 cc. portions of this solution and the total volume made up to 100 cc. The conductivity and digestion of the solution were then determined as described for the egg albumin.

The Optimum Hydrogen Ion Concentration for Pepsin Digestion.

The optimum hydrogen ion concentration for the activity of pepsin has been determined many times. All the methods used for following the digestion, however, have depended on the change in some physical property of the protein. It seemed of interest therefore to determine the optimum degree of acidity for the reaction when the hydrolysis was followed by means of the increase in amino nitrogen, which

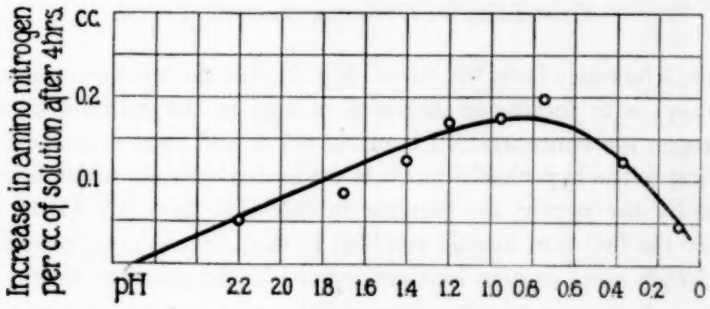


FIG. 2. Influence of pH on the rate of digestion of egg albumin.

probably represents correctly the actual course of the digestion. The method has the disadvantage, however, that only comparatively large changes can be followed. The results of an experiment made with egg albumin solutions of different pH (adjusted with HCl) are given in Fig. 2.

The time of digestion was 4 hours. The figure shows that the optimum acidity for the digestion as determined by the increase in amino nitrogen is at about pH 1.0 (0.1 N). This is slightly more acid than that found by Sörensen,[1] Michaelis and Mendelssohn,[23] or Okada,[24] and much more acid than that found by Ringer.[15] It must be remembered,

[23] Michaelis, L., and Mendelssohn, A., *Biochem. Z.*, 1914, lxv, 1.
[24] Okada, S., *Biochem. J.*, 1916, x, 126.

however, that the chemical changes followed by the increase in amino nitrogen represent much more complete hydrolysis than those followed by the other authors. The curve therefore probably does not represent the correct optimum for the digestion of the protein itself but probably also the digestion of some of the primary products. The careful work of Ringer has shown that the optimum zone for the digestion of these products extends further to the acid side than the zone for the digestion of the protein itself. This probably accounts for the difference in the optimum found by the different methods and agrees with the results of Sörensen[1] who found that the optimum shifts to the acid side with more complete digestion.

The Effect of Adding Salt with a Common Ion to a Solution Already Containing the Optimum Amount of Acid.

It will be noted from the curve (Fig. 2) that the amount of digestion increases with increasing amounts of acid in the solution until the hydrogen ion concentration is about 0.1 N and then decreases. According to the hypothesis that it is the ionized protein which is hydrolyzed by the pepsin, the increase in digestion from pH 4.0 to 1.0 is due to the fact that as acid is added to the albumin more protein salt and hence more protein ions are formed in the solution, until all the albumin is present as salt. The addition of a further amount of acid serves to depress the concentration of protein ions again due to the effect of the common ion. According to this mechanism the hydrogen ion concentration is the determining factor on the alkaline side of the optimum while on the acid side the concentration of the anion is the determining factor. It can be predicted therefore that if a solution of a salt (having the same anion as the acid) is added to a solution of the protein which already contains the optimum amount of acid, the depressing effect of the salt on the digestion should be the same as if excess acid had been added, provided the final anion concentration is the same. The conductivity of the albumin salt should also be diminished. In the case of egg albumin this cannot be experimentally verified owing to the fact that the albumin precipitates under these conditions, and also since the conductivity of the protein in such strongly acid solutions is so small, compared to

the total conductivity, as to render the measurement very uncertain. It will be shown later, however, that in the case of gelatin the decrease in conductivity can be followed and is proportional to the decrease in the rate of digestion.

TABLE I.

Increase in Amino Nitrogen per Cc. of Solution Containing Normal Total Chlorine Concentration Furnished by Different Salts.

Original solution 0.5 N HCl.
Made up to 1.0 N chloride concentration with salt noted.

Salt.	pH	Increase in NH_3 nitrogen per cc after 6 hrs. at 25°C.
		cc.
O	0.42	0.25
		0.26
NaCl	0.40	0.15
		0.13
KCl	0.42	0.15
		0.15
$CaCl_2$	0.41	0.17
		0.15
$MgCl_2$	0.42	0.12
		0.11
$SrCl_2$	0.42	0.17
		0.18
$AlCl_3$	0.40	0.10
		0.11
HCl	0.13	0.13
		0.14

Table I contains a summary of the results of such an experiment in which a series of egg albumin solutions all containing a total chlorine ion concentration of 0.5 N and at a pH of 0.42 were brought to a total chlorine ion concentration of 1.0 N by the addition of the salts noted or excess acid. The final solutions therefore were all

1.0 N in respect to the chlorine ion but those which had been brought to this concentration by the addition of salts were of course much less acid than the one to which excess acid had been added. The amount of digestion in all the solutions containing the same chlorine ion concentration was approximately the same, however. This result indicates that the controlling factor on the acid side of the optimum is the anion concentration and not the hydrogen ion concentration. As a corollary of this it can be stated that the addition of salt to a protein solution will cause the optimum hydrogen ion concentration for digestion to be shifted to the alkaline side. This was the effect noted by Michaelis and Mendelssohn.[23]

The above question has recently been examined by Gyemant.[25] This author found, however, the optimum pH for digestion remained at about pH 2.0 even though the anion concentration was the same in all the solutions. He concludes therefore that the decrease in the rate of digestion on the acid side of the optimum is due to the influence of the hydrogen ion on the pepsin as proposed by Michaelis.

The experiments described in this paper are complicated by the fact that the egg albumin was partially precipitated by the high concentrations of salt and acid used. This may account for the difference between the present results and those of Gyemant. The discrepancy may also be due to the fact that Gyemant followed the reaction by means of the increase in non-protein nitrogen whereas the author used the increase in amino nitrogen. In view of Gyemant's results and of the complicating factor of precipitation in the present experiments, they cannot be considered as conclusive evidence in favor of the view that the anion alone affects the digestion on the acid side of the optimum. It is possible that both ions are active. It appears to the author, however, that the action is exerted on the protein rather than the enzyme in view of the fact that different proteins show slightly different optimum pH, and of the close connection between the conductivity and rate of digestion of gelatin solutions (as described below in this paper).

The Conductivity and Rate of Digestion of Gelatin Solutions.

It was mentioned above that determinations of the conductivity of egg albumin solutions in strongly acid solution were made uncertain owing to the precipitation of the protein. This difficulty is not encountered with gelatin. Gelatin possesses the further advantage that the rate of digestion in the very early stages may be easily fol-

[25] Gyemant, A., *Biochem. Z.*, 1920, cv, 155.

lowed by noting the time necessary to cause a certain degree of liquefaction of the gelatin.

A series of gelatin solutions, containing 5 per cent dry weight of gelatin and adjusted to various hydrogen ion concentrations by means of HCl, were prepared. The gelatin had previously been purified as described by Loeb.[26] The conductivity of the solutions and the time necessary for them to reach an easily determined degree of liquefaction were then determined. The reciprocal of this time is plotted in the curve as the rate. Fig. 3 and Table II show the result of a typical experiment of this kind. It is clear that the rate of diges-

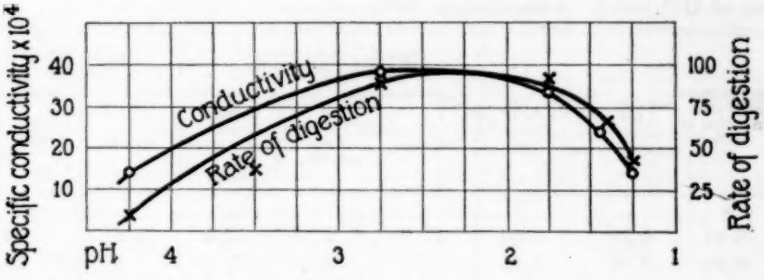


FIG. 3. Influence of pH on the rate of digestion and conductivity of gelatin solutions.

tion and the conductivity of the solution have their maximum value at the same hydrogen ion concentration, and that the curves are nearly parallel throughout. The rate of digestion decreases slightly more rapidly than the conductivity of the solution on the alkaline side of the optimum and slightly less rapidly on the acid side. This peculiarity was noted in all the experiments made and can hardly be ascribed to experimental errors. It shows that the digestion on the alkaline side of the optimum is slightly less rapid than would be predicted from the conductivity data and that it is slightly more rapid on the acid side. The divergence on the acid side is due to the fact that in such strongly acid solutions the acid itself has some action on the protein as was shown by control experiments without any pepsin. The correction is too uncertain to be applied to the figures, however.

[26] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

It can only be said that such a correction is necessary and that it would be in the right sense. The divergence on the alkaline side is probably due to the fact that the amount of hydrolysis selected as the end-point represented too great a percentage change in the original substrate concentration to assume that the substrate concentration remained constant during the course of the experiment.

TABLE II.

pH, Conductivity, and Rate of Digestion of Gelatin Solutions.

Gelatin, 5 per cent dry weight in solution of total (approximate) concentration of HCl noted. Temperature, 37°C.

Approximate total concentration of HCl.	pH	$C_H \times 10^4$	Specific conductivity of (Reciprocal ohms × 10^4.)			Time for gelatin to liquefy.	
			Solution.	HCl of same pH as solution.	Gelatin chloride (= κ solution − κHCl).	Hours.	Rate = $\frac{40}{\text{hrs.}}$
N							
0.02	4.23	0.60	17.2	0.27	17.0	4.5	9
0.04	3.50	3.16	33.1	1.47	31.6	1.1	36
0.06	2.78	16.6	48.2	7.7	40.5	0.40	100
0.08	1.78	166.0	110.0	76.0	34.0	0.42	95
0.10	1.48	331.0	175.0	151.0	24.0	0.65	62
0.12	1.26	550.0	260.0	245.0	15.0	0.92	43

EXPERIMENTAL.

50 gm. (dry weight) of purified isoelectric gelatin were dissolved in warm water and the volume was made up to 500 cc. Increasing amounts of HCl were then added to a series of 50 cc. portions of this solution and the volume of each portion was then made up to 100 cc. 2 cc. of 2 per cent pepsin solution were then added to 75 cc. of each of the above solutions and the solutions put in the water bath at 37°C. At short intervals 5 cc. samples were pipetted from each of the solutions into a series of test-tubes containing 2 cc. of water. These tubes were then placed in a water bath at 2°C. for 10 minutes, taken out, and the degree of liquefaction was compared with that of a standard tube. (This is a slight modification of the method of Fermi as described by Dernby.[27]) This procedure was repeated until a sample from each of the tubes showed the same degree of liquefaction as the standard tube. In this way the time necessary to produce a certain degree of liquefaction can be accurately and easily determined. The pH and con-

[27] Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

ductivity of the solution were determined on the remaining 25 cc. of solution to which had been added the equivalent amount of inactivated pepsin. The determinations were made as described above except that the measurements were made at 37°C.

The Combination of Pepsin and Gelatin.

In a former paper[28] it was shown that the amount of pepsin which combined with a given quantity of coagulated egg albumin depended entirely on the reaction of the solution in which the egg albumin was suspended. The greatest amount of pepsin was combined when the

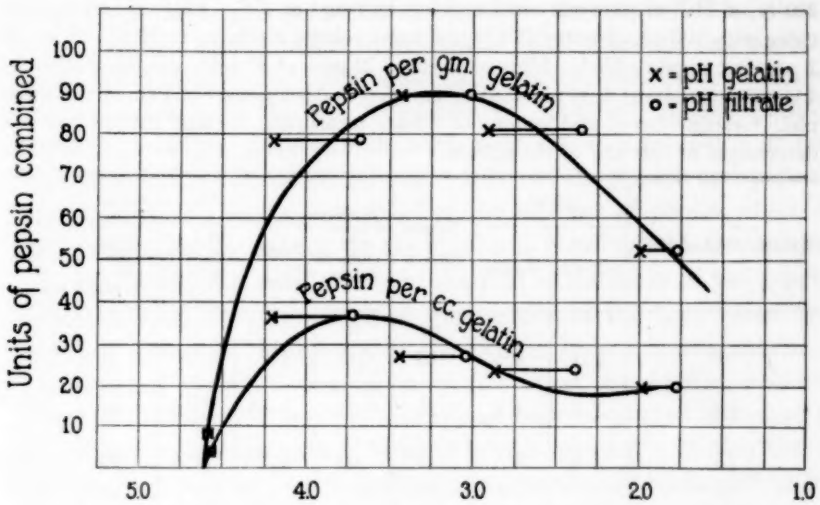


FIG. 4. Influence of pH on the combination of pepsin and gelatin.

solution had a reaction of pH 2.5 to 3.0. It was pointed out that this was probably part of the mechanism that caused insoluble proteins to digest more rapidly at this reaction than at any other since it seems that the rate of digestion must depend on the amount of pepsin in the solid protein.

These experiments have been repeated with gelatin and show in general the same result. The results of such an experiment are given in Fig. 4 and Table III. The figures show that a greater amount

[28] Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 113.

of gelatin and pepsin is combined at about pH 3.0 than in either more or less acid solutions. In the case of gelatin the volume varies greatly with the reaction owing to the effect of the acid on the swelling of gelatin. The swelling is greatest at about pH 3.4 (*cf.* Loeb[20]). It might be supposed therefore that more pepsin was combined with the gelatin at about this degree of acidity simply because there was

TABLE III.

Combination of Gelatin and Pepsin.

5 gm. of isoelectric purified gelatin (= 0.75 gm. of dry weight) suspended in 200 cc. of HCl of strength noted and left 16 hours at 2°C. Filtered and washed twice with 100 cc. of water (5°C.) and total volume made up to 75 cc. 5 cc. of 2 per cent pepsin added. Allowed to stand 20 min. at 5° with occasional stirring. 4 cc. of supernatant fluid pipetted off and pepsin determined* in 1 cc. of this sample. Gelatin filtered and volume of filtrate measured. Gelatin melted and pH determined of this and of the filtrate.

Concentration of HCl.	pH of		Volume of filtrate.	Volume of gelatin (= 80 − volume of filtrate).	Units of pepsin.			
	Filtrate.	Gelatin.			Pepsin per cc. of filtrate.	Total pepsin in filtrate (*a*).	Total pepsin in gelatin (115–*a*).	Pepsin per 10 cc. of gelatin.
			cc.	*cc.*				
0	5.3	4.6	63	17	1.7	107.0	8	5
$\frac{M}{256}$	3.6	4.2	57	23	0.65	37.0	78	34
$\frac{M}{64}$	3.0	3.4	47	33	0.52	25.0	90	27
$\frac{M}{8}$	2.4	2.9	47	33	0.71	33.0	82	25
$\frac{M}{4}$	1.8	2.0	53	27	1.17	62.0	53	20
0 Control. No gelatin.	5.2		80	0	1.44	115.0	0	0

* *Cf.* Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113. The relative amount of pepsin is taken as the reciprocal of the time in hrs. required to cause a 5 per cent change in the conductivity of a 5 per cent egg albumin solution titrated to pH 1.7 with hydrochloric acid when 1 cc. of pepsin solution is added to 25 cc. of egg albumin at 37°C. The unit of pepsin is taken as that amount which when dissolved in 1 cc. and added to 25 cc. of the egg albumin solution will cause a change of 5 per cent in the conductivity in 1 hr.

a greater volume of gelatin present at this point. It will be seen, however, from Table II and Fig. 3 that this is not true since the figures show that there is a maximum even when the results are calculated to the basis of pepsin per cubic centimeter of gelatin. That is, there is not only more pepsin combined with a gram of gelatin at this pH but also the concentration of the pepsin in the gelatin is greatest here. There is considerable uncertainty as to the pH measurements since, as the table shows, the reaction of the liquid was always considerably more acid than that of the gelatin. In most of the experiments the difference was much more marked than in the experiment given; in some cases the maximum fell at about pH 2.2. This agrees much more closely with the optimum acidity found for digestion and for the ionization of the protein. Owing to the uncertainty of the pH measurement, however, it is probably better to make no definite statement as to the exact position of the optimum acidity for the combination between the gelatin and pepsin. The determining factor in regulating the amount of pepsin which is combined with the gelatin is the chemical condition of the gelatin and pepsin and not a difference in the rate of diffusion of the pepsin since the same curve is obtained irrespective of the time (after the first few minutes) during which the gelatin is left in the solution. The simplest explanation would seem to be that the gelatin combines only with the ionized protein and the amount combined therefore is dependent on the amount of ionized gelatin present. Pepsin therefore behaves just as do the inorganic anions studied by Loeb[20] as far as the influence of the hydrogen ion concentration on the combination is concerned.

DISCUSSION AND SUMMARY.

The experiments described above show that the rate of digestion and the conductivity of protein solutions are very closely parallel. If the isoelectric point of a protein is at a lower hydrogen ion concentration than that of another, the conductivity and also the rate of digestion of the first protein extends further to the alkaline side. The optimum hydrogen ion concentration for the rate of digestion and the degree of ionization (conductivity) of gelatin solutions is the same, and the curves for the ionization and rate of digestion as

plotted against the pH are nearly parallel throughout. The addition of a salt with the same anion as the acid to a solution of protein already containing the optimum amount of the acid has the same depressing effect on the digestion as has the addition of the equivalent amount of acid. These facts are in quantitative agreement with the hypothesis that the determining factor in the digestion of proteins by pepsin is the amount of ionized protein present in the solution. It was shown in a previous paper[11] that this would also account for the peculiar relation between the rate of digestion and the concentration of protein. The amount of ionized protein in the solution depends on the amount of salt formed between the protein (a weak base) and the acid. This quantity, in turn, according to the hydrolysis theory of the salts of weak bases and strong acids, is a function of the hydrogen ion concentration, up to the point at which all the protein is combined with the acid as a salt. This point is the optimum hydrogen ion concentration for digestion, since the solution now contains the maximum concentration of protein ions. The hydrogen ion concentration in this range therefore is merely a convenient indicator of the amount of ionized protein present in the solution and takes no active part in the hydrolysis. After sufficient acid has been added to combine with all the protein, *i.e.* at pH of about 2.0, the further addition of acid serves to depress the ionization of the protein salt by increasing the concentration of the common anion. The hydrogen ion concentration is, therefore, no longer an indicator of the amount of ionized protein present, since this quantity is now determined by the anion concentration. Hence on the acid side of the optimum the addition of the same concentration of anion should have the same influence on the rate of digestion irrespective of whether it is combined with hydrogen or some other ion (provided, of course, that there is no other secondary effect of the other ion). The proposed mechanism is very similar to that suggested by Stieglitz and his coworkers[29] for the hydrolysis of the imido esters.

Pekelharing and Ringer[5] have shown that pure pepsin in acid solution is always negatively charged; *i.e.*, it is an anion. The experiments described above show further that it behaves just as would be expected of any anion in the presence of a salt containing the protein ion as the cation and as has been shown by Loeb[20] to be the case with inorganic anions.

[29] Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix. 29, 164, 402, 437, 586, 719

Nothing has been said in regard to the quantitative agreement between the increasing amounts of ionized protein found in the solution (as shown by the conductivity values) and the amount predicted by the hydrolysis theory of the formation of salts of weak bases and strong acids. There is little doubt that the values are in qualitative agreement with such a theory. In order to make a quantitative comparison, however, it would be necessary to know the ionization constant of the protein and of the protein salt and also the number of hydroxyl (or amino) groups in the protein molecule as well as the molecular weight of the protein. Since these values are not known with any degree of certainty there appears to be no value at present in attempting to apply the hydrolysis equations to the data obtained.

It it clear that the hypothesis as outlined above for the hydrolysis of proteins by pepsin cannot be extended directly to enzymes in general, since in many cases the substrate is not known to exist in an ionized condition at all. It is possible, however, that ionization is really present or that the equilibrium instead of being ionic is between two tautomeric forms of the substrate, only one of which is attacked by the enzyme. Furthermore, it is clear that even in the case of proteins there are difficulties in the way since the pepsin obtained from young animals, or a similar enzyme preparation from yeast or other microorganisms, is said to have a different optimum hydrogen ion concentration than that found for the pepsin used in these experiments. The activity of these enzyme preparations therefore would not be found to depend on the ionization of the protein. It is possible of course that the enzyme preparations mentioned may contain several proteolytic enzymes and that the action observed is a combination of the action of several enzymes. Dernby[27] has shown that this is a very probable explanation of the action of the autolytic enzymes. The optimum hydrogen ion concentration for the activity of the pepsin used in these experiments agrees very closely with that found by Ringer for pepsin prepared by him directly from gastric juice and very carefully purified. Ringer's pepsin probably represents as pure an enzyme preparation as it is possible to prepare. There is every reason to suppose therefore that the enzyme used in this work was not a mixture of several enzymes.

RADIOACTIVITY AND PHYSIOLOGICAL ACTION OF POTASSIUM.

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I. INTRODUCTION.

The cations Na, K, and Ca are essential constituents of physiologically balanced salt solutions such as blood serum, tissue fluids, and sea water, and, in the absence of one of these ions, physiological processes cannot, as a rule, occur for any great period of time. Zwaardemaker[1] has recently advanced the interesting idea that the indispensability of potassium in cardiac action is due to the slight radioactivity of that element. To prove this idea, he has demonstrated that other radioactive substances, *e.g.* thorium, uranium, ionium, radium, etc., can replace the K ion in restoring heart beat after the heart has stopped beating in a Ringer solution containing no potassium. The most significant fact demonstrated by Zwaardemaker and his collaborators is that for the replacement of potassium by other radioactive substances, equiradioactive doses are required. Zwaardemaker's conclusion that the action of potassium in physiologically balanced salt solutions is a result of its radioactivity is of such great importance that it seemed justifiable to test the applicability of this view to physiological processes other than the heart beat.

As a result of experiments by Herbst[2] and others, it is known that sea urchin eggs are unable to develop when placed in potassium-free sea water immediately after fertilization, and Herbst found that rubidium and cesium could replace potassium to a limited extent. It seemed to us that a further study of the replacement of K by cesium

[1] Zwaardemaker, H., *J. Physiol.*, 1919–20, liii, 273.

[2] Herbst, C., *Arch. Entwcklngsmechn. Organ.*, 1901, xi, 617.

and certain radioactive substances might help in deciding definitely whether or not the physiological influence of potassium is dependent on its radioactive properties.

II. EXPERIMENTAL.

The method used in this work was as follows. Eggs of *Arbacia* were fertilized in potassium-free artificial sea water of the following composition: 100 cc. of M/2 NaCl + 7.8 cc. of M/2 $MgCl_2$ + 3.8 cc. of M/2 $MgSO_4$ + 1.75 cc. of M/2 $CaCl_2$. To this solution enough M/10 $NaHCO_3$ (usually 0.2 cc. per 25 cc. of solution) was added to bring the pH to between 7.4 and 8.0. The chemicals used were Kahlbaum's purest preparations. Both glass- and metal-distilled water were used in preparing the solutions. After fertilization, the eggs were washed three times in similar K-free artificial sea water and were then placed in various solutions to observe development. In all experiments, some of the fertilized eggs were placed in (*a*) normal sea water, and (*b*) alkaline K-free sea water for control. In no case where eggs were placed in K-free sea water did the development go beyond the sixteen cell stage, and in a few hours the eggs were disintegrated. All sea water controls developed into normal plutei.

The criterion used in these experiments for the adequacy of the substitutes for potassium was the formation of normal swimming blastulæ within 24 hours. In reality development went beyond this stage in most instances.

A. Potassium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of K-free sea water to which was added enough KCl to make the total concentration of KCl in the dishes M/4,600, M/2,300, M/1,300, M/850, M/660, M/550, M/470, M/370, M/330, etc., up to M/18, the latter concentration of KCl being about 5.5 times that present in normal sea water. The pH in these and succeeding experiments was about 7.4.

From Table I we see that fertilized eggs do not form swimming larvæ in artificial sea water with a concentration of KCl lower than M/660. We also see that a certain excess of potassium, *i.e.* 5.5 times its normal concentration in sea water, does not interfere with development up to the gastrula stage.

B. Rubidium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of K-free sea water to which was added enough RbCl to make the total concentration of RbCl in the dishes M/4,600, M/2,300, M/1,300, M/850, M/660, M/550, M/470, M/370, M/330, etc., to M/18.

The experiments proved that RbCl is entirely able to replace KCl in the development of swimming blastulæ and that the minimal concentration of RbCl needed is M/660, approximately that needed when KCl is used.

TABLE I.

Concentration of Potassium Needed for Development.

Molecular concentration of KCl.	Stage of development after	
	5 hrs.	24 hrs.
0	2–16 cells disintegrating.	Dead.
M/4,600	2–16 " "	"
M/2,300	2–16 " "	"
M/1,300	Few as far as 64 cells, mostly disintegrating.	"
M/850	" " " " 64 " " "	"
M/660	" " " " 64 " " "	Few swimming gastrulæ.
M/550	Some disintegrating, many good early blastulæ.	Swimming gastrulæ.
M/470	Almost all early blastulæ.	All gastrulæ.
M/370	" " " "	" "
M/330	All early blastulæ.	" "
M/150	" " "	" "
M/125	" " "	" "
M/100	" " "	" "
M/52	" " "	" "
M/27	" " "	" "
M/18	" " "	" "

C. Cesium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of potassium-free sea water to which was added enough CsCl to make the total concentration of CsCl in the dishes M/4,600, M/2,300, M/1,300, M/1,000, M/850, M/660, M/500, M/250, M/125, etc., to M/18.

From Table II we see that CsCl can replace potassium chloride and that the minimal molecular concentration required for this purpose is practically identical for both salts. If the action of potassium is

due to its radioactivity, we should have to conclude that cesium has the same degree of radioactivity as potassium which is contrary to the facts known at present. Development with CsCl is possibly slower than with KCl, and the development does not go so far as in

TABLE II.

Concentration of Cesium Needed for Development.

Molecular concentration of CsCl.	Stage of development after	
	6 hrs.	24 hrs.
0	2–16 cells disintegrating.	Dead.
M/4,600	All disintegrating.	"
M/2,300	8–16 cells disintegrating.	"
M/1,300	8–16 " "	"
M/1,000	Few 16–32 cells, most disintegrating.	"
M/850	Few 64 cells, most disintegrating.	"
M/660	" 64 " " "	"
M/500	About 50 per cent in 64 cell stage.	About 50 per cent very slowly swimming blastulæ.
M/250	Most in 64 or 128 cell stage.	About 50 per cent slowly swimming blastulæ.
M/170	Practically all 128 cells.	Still more swimming blastulæ.
M/125	" " 128 "	Mostly rapidly swimming blastulæ; few gastrulæ.
M/100	" " 128 "	Most blastulæ; some rapidly swimming gastrulæ.
M/80	" " 128 "	Most blastulæ; some rapidly swimming gastrulæ.
M/64	" " 128 "	Same, but more gastrulæ.
M/44	" " 128 "	Swimming blastulæ, many degenerating.
M/33	" " 128 "	Mostly slowly swimming blastulæ.
M/18	" " 128 "	Mostly swimming gastrulæ.

the case of KCl probably on account of the greater toxicity of cesium which in rather low concentrations kills the larvæ.

D. Thorium Chloride Experiments.—It seemed important to determine whether or not potassium could be replaced by a radioactive element like thorium. Zwaardemaker found that in winter a solu-

tion of M/10,000 and in summer about M/100,000 thorium nitrate could replace potassium in his experiments on the heart. In our experiments, we fertilized eggs as described and after thorough washing in K-free sea water they were placed in dishes containing 25 cc. of K-free sea water and enough thorium chloride to make the total concentration of $ThCl_4$ in the dishes M/200,000, M/100,000, M/66,000, M/33,000, M/25,000, M/21,000, M/11,000, M/7,500, M/4,800, M/3,300, and M/2,100. To these solutions, which were quite acid, enough M/10 $NaHCO_3$ was added to bring the pH between 7.2 and 8.0. In all these

TABLE III.

Effect of Thorium in Replacing Potassium.

Molecular concentration of $ThCl_4$.	Stage of development after	
	4 hrs.	24 hrs.
0	2–8 cell stage disintegrating.	Dead.
M/200,000	2–8 " " "	"
M/100,000	2–8 " " "	"
M/66,000	2–8 " " "	"
M/33,000	2–8 " " "	"
M/25,000	2–8 " " "	"
M/21,000	2–8 " " "	"
M/11,000	2–8 " " "	"
M/7,500	2–8 " " "	"
M/4,800	2–8 " " "	"
M/3,300	2–8 " " "	"
M/2,100	2–8 " " "	"

cases the Th was probably in suspension and not in true solution and in the three highest concentrations of $ThCl_4$ addition of bicarbonate caused visible precipitation. Since Zwaardemaker's Ringer solutions were also slightly alkaline the Th in his solutions was also probably in suspension, but this would not interfere with the radioactivity since the radioactivity depends on the changes in the nucleus of an atom and since these nuclear changes do not depend on whether or not the substance is in true solution. As is seen in Table III in no case where Th was used to replace K did we observe the development of swimming blastulæ and in all dishes disintegration occurred before the sixteen cell stage was reached just as in the K-free sea water

control. It was shown that death in the thorium experiments was due to lack of K and not to the toxicity of $ThCl_4$, because when $ThCl_4$ was added to normal sea water in the same concentrations in which it was used to replace KCl it in no way interfered with the formation of normal larvæ, owing probably to the fact that the Th was in suspension and not in true solution. The radioactivity of the $ThCl_4$ was not sufficient to interfere with the development of the eggs up to the pluteus stage at least.

E. Uranium Acetate Experiments.—A series of experiments with the radioactive element uranium also gave negative results; *i.e.*, in no case were swimming larvæ obtained where uranium acetate was used to replace potassium and the eggs died before the sixteen cell stage was reached. The concentrations of uranium acetate used in the K-free sea water were M/125,000, M/62,500, M/42,000, M/32,000, M/25,000, M/16,000, M/12,500, M/11,000, M/6,800, M/4,700, M/3,000, and M/2,100. The pH in these solutions was brought to about 7.6 through the addition of $NaHCO_3$ and in the two highest concentrations some uranium was visibly precipitated. The lack of development of the eggs here was not due to the toxicity of the uranium salt because, when the uranium acetate was added to sea water in the same concentrations, normal larvæ developed except in the two highest concentrations and here the pH was found to be below 7. The uranium as well as the thorium was probably in suspension in the sea water and not in true solution but this, of course, did not influence the radioactivity. It is of interest to note that, here and in the thorium experiments, the radioactive elements did not inhibit the action of K as Zwaardemaker seems to assume.

III. Theoretical Remarks.

It appears from the foregoing experiments that in the development of the fertilized egg of *Arbacia* up to the blastula-gastrula stage potassium can be replaced by the non-radioactive Cs ion, the minimum concentration required for development being practically the same for both cations (KCl minimum concentration = M/660, CsCl minimum concentration = M/500). Furthermore, it is apparent that K cannot be replaced in our experiments by suspensions of the radioactive elements thorium and uranium. Hence, the action of potassium in the

development of the eggs of *Arbacia* cannot be attributed to its very slight radioactivity.

These results would seem at variance with the findings of Zwaardemaker and his coworkers in their experiments on the heart were it not for the fact that they also were able to replace potassium by cesium, which Zwaardemaker designates as "physiologically but not physically" radioactive. As a matter of fact nobody has thus far shown that Cs possesses more radioactivity than any other non-radioactive element; *e.g.*, Na. Zwaardemaker's observation that a heart which has ceased to beat in a solution lacking KCl can be resuscitated by radioactive substances may be explained without the assumption that K acts through its radioactivity. Lingle[3] found that when the ventricle of the heart of the turtle was suspended in a moist chamber where the air was replaced by pure oxygen the strip was able to beat for a considerable time, as long as 3 days, after the beats had been initiated by submersing the ventricle for a short time in a pure NaCl solution. The beats continued in some cases until the putrefaction of the heart tissues put a stop to them. This experiment proves that the KCl is not needed to provide the stimulus for the heart beat but that it is only needed to counteract some of the toxic effects of a pure NaCl solution when the heart is submersed in such a solution. Lingle found, moreover, that the heart which had stopped beating in a pure solution of NaCl began to beat again when 10 cc. of 3 per cent H_2O_2 were added to 90 per cent of the isotonic solution of NaCl. He could show by control experiments that only the bubbles of O_2 with which the muscle became frosted were responsible for the resuscitation. The ventricle thus resuscitated could then continue to beat for many hours. Lingle suggests that the oxygen supply in a solution is inadequate for a heart beating in a pure solution of NaCl unless a more rapid source of supplying oxygen than mere diffusion from the air is provided. We do not know how the increased supply of oxygen can resuscitate the heart which had ceased to beat in a pure solution of NaCl but the tentative suggestion may be permitted that this effect is due to the transformation of a harmful substance formed during the activity of the heart in the absence of K, or Ca, or

[3] Lingle, D. J., *Am. J. Physiol.*, 1902–03, viii, 75.

both, into a less harmful one through a more rapid oxidation. Lind has shown that penetrating rays can cause the formation of water from O and H and that H_2O_2 seems to be an intermediate product in the reaction.[4] On the basis of these observations it is quite probable that H_2O_2 is formed or that oxygen is activated in some other form when penetrating rays go through the cells of the heart, and that the rate of oxidation is increased in the cells of the heart. This might explain Zwaardemaker's experiments on the restoration of the heart beat by U, Th, and radium without compelling us to assume that KCl, Rb, and even Cs act physiologically by radioactivity.

CONCLUSIONS.

1. The non-radioactive cesium ion can replace the potassium ion almost quantitatively in solutions required for the development of the egg of the sea urchin into swimming blastulæ.

2. Thorium chloride and uranium acetate cannot replace the potassium chloride in the solutions required for the development of the egg.

3. Thorium chloride and uranium acetate do not antagonize the action of the potassium contained in sea water upon the development of eggs.

[4] Lind, S. C., *J. Am. Chem. Soc.*, 1919, xli, 551.

CHEMICAL CHARACTER AND PHYSIOLOGICAL ACTION OF THE POTASSIUM ION.

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I. INTRODUCTION.

Zwaardemaker[1] has recently made the interesting suggestion that the rôle of potassium in physiologically balanced salt solutions—*e.g.* the blood or the sea water—is due to the very slight radioactivity of this element and not to its chemical character as determined by its position in the series of elements. It has been pointed out by R. F. Loeb[2] in another paper in this *Journal* that K cannot be replaced by Th and U as far as the development of the sea urchin egg is concerned, that the non-radioactive element Cs is capable of replacing potassium to some extent in this case, and that Zwaardemaker's observations on the influence of radioactive substances on the heart beat might be explained without the assumption that the physiological action of K is due to its radioactivity.

This then suggests that the physiological action of potassium is due to its chemical character. We know through the work of Sir Ernest Rutherford that radioactivity is caused by an explosive charge in the nucleus of the atom while the chemical and most of the physical properties of the atom depend upon its external ring or shell of electrons. These latter properties are repeated periodically in the series of elements arranged by their atomic numbers and if we can show that the physiological action of an element corresponds to its position in the periodic table we know that we are dealing with purely chemical effects and not with radioactive effects. We intend to show in an indirect way that the action of K in physiologically balanced salt

[1] Zwaardemaker, H., *J. Physiol.*, 1919–20, liii, 273

[2] Loeb, R. F., *J. Gen. Physiol.*, 1920–21, iii, 229.

solutions corresponds to its purely chemical character; *i.e.*, its position in the periodic table (or rather to its atomic number and arrangement of external electrons).

II. The Resemblance in the Antagonistic Effects of the NH_4 and K Ions.

According to their physiological action the ions of the alkaline metals can be arranged in two distinct groups, the one including Li and Na, the other K, Rb, and Cs. The difference in the two groups is noticeable in various phenomena. Isotonic solutions of LiCl and NaCl will give rise to muscular twitchings while KCl, RbCl, and probably CsCl will not. Experiments on the egg of *Fundulus* show that toxic solutions of salts with bivalent metals, such as $MgCl_2$ or $CaCl_2$, can be rendered less toxic by the addition of KCl, RbCl, or CsCl, but not or practically not by the addition of NaCl or LiCl.[3] It is known that the NH_4 ion resembles in its chemical behavior the K ion more closely than it does the sodium ion, and Langmuir[4] has utilized this fact in support of his theory of the cubical atom. If it be true that the physiological action of K depends upon its chemical character, the close resemblance between the chemical character of NH_4 and K should express itself in phenomena of antagonism. NH_4Cl is generally very toxic for cells but it can be used with good effect in experiments on the egg of *Fundulus* which is surrounded by a rather impermeable membrane. In experiments on *Fundulus* it can be shown that the antagonistic action of the NH_4 ion is like that of the members of the K group and not like that of the members of the Na group.

When newly fertilized eggs of *Fundulus* are put into a 5 M/32 solution of $CaCl_2$ practically no egg (*i.e.* less than 2 per cent of the eggs) can form an embryo. When the 5 M/32 $CaCl_2$ solution is made up in solutions of different chlorides instead of in H_2O it is found that in LiCl and NaCl the toxicity of the $CaCl_2$ solution is not diminished. When, however, the 5 M/32 $CaCl_2$ solution is made up in KCl, RbCl, CsCl, or NH_4Cl a considerable percentage of the eggs can develop into embryos as is shown in Table I.

[3] Loeb, J., *J. Biol. Chem.*, 1914, xix, 431.

[4] Langmuir, I., *J. Am. Chem. Soc.*, 1920, xlii, 274.

The same fact can be demonstrated equally well with other toxic solutions; *e.g.*, Na_3 citrate. If newly fertilized eggs of *Fundulus* are put into M/100 Na_3 citrate practically no egg can form an embryo, and the result remains the same if the M/100 solution of Na_3 citrate is made up in different concentrations of LiCl or NaCl. When, however, the M/100 solution of Na_3 citrate is made up in KCl, RbCl, CsCl, or NH_4Cl a considerable number of eggs develop into embryos as indicated in Table II.

TABLE I.

	Percentage of newly fertilized *Fundulus* eggs which can form embryos in 5 M/32 $CaCl_2$ when this solution is made up in						
	0	M/80	M/40	M/20	M/10	M/5	3 M/10
LiC.....	1.5	0	0	0	0	0	0
NaCl.....		1	2	1	0	0	0
KCl.....		5	21	21	44	60	64
RbCl.....		19	23	26	40	54	43
CsCl.....		3	4	14	9	17	30
NH_4Cl.....		1	0	3	4	17	16

TABLE II.

	Percentage of eggs of *Fundulus* which can develop in M/100 sodium citrate solution if this solution is made up in					
	0	M/40	M/20	M/10	M/5	3 M/10
LiCl.....	0	3	2	0	0	0
NaCl.....	0	6	0	2	1	0
KCl.....	0	26	19	57	52	53
RbCl.....	8	46	55	60	55	42
CsCl.....	8	40	60	56	32	
NH_4Cl.....	8	2	1	2	45	36

The table shows that when the M/100 Na_3 citrate solution is made up in M/5 KCl, RbCl, CsCl, or NH_4Cl practically half the eggs form embryos. In this experiment the addition of the Cl ion may diminish the toxicity of the citrate solution but if this be true the fact remains that the Cl ion can have this effect only when it is added with K or NH_4 ions and not when added with Na or Li ions.

The NH_4 ion, therefore, resembles in its physiological behavior the K ion more than it does the Na or Li ion.

III. The Antagonism between Li and K.

In any series of ions based on their chemical or physical behavior Na occupies a position between Li and K. Li has a smaller and K has a larger ionic radius than Na. If we replace some of the Na ions of sea water by Li ions we alter the properties of the solution in one sense, and if we replace part of the Na ions by K ions we alter the properties in the opposite sense. We should, therefore, expect that if we replace a certain percentage of Na ions in sea water by Li ions the toxic character of the solution should be diminished if we replace at the same time also a certain percentage of the Na ions by K ions; since with the combined increase of the K ions and of Li ions the effect of Na ions might be more nearly approximated.

The newly fertilized egg of the sea urchin (*Arbacia*) can develop into gastrulæ in an "artificial sea water" of the following composition.

100.0 cc. of M/2 NaCl
1.75 cc. of M/2 $CaCl_2$
2.2 cc. of M/2 KCl
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$

To this was added 0.8 cc. of M/10 $NaHCO_3$ to bring the artificial sea water to a pH of about 7.4.

We prepared the following solution in which M/2 NaCl of the artificial sea water was replaced by M/2 LiCl and which was free from K. Its composition was:

100.0 cc. of M/2 LiCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $NaHCO_3$

This solution, which we will call the Li mixture, permitted us to replace the Na in natural or artificial sea water by Li without altering the constitution of the sea water in any other direction except in regard to K, the concentration of which it was our intention to vary in the experiments.

Our first experiments consisted in mixing various quantities of natural sea water and Li mixture to find out the maximal amount of Li in natural sea water which still permitted the formation of normal blastulæ in about 16 or 20 hours; the eggs were put into the solution immediately after fertilization. It was found that only 8 per cent of the Li mixture could replace the natural sea water without preventing the development of the eggs into swimming blastulæ. When, however, the proportion of KCl contained in the sea water was increased thirteen times its normal amount the eggs were able to develop into larvæ when as much as 52 per cent of Na was replaced by Li. It is therefore possible to increase the tolerance of the sea urchin egg

TABLE III.

Maximal Amount of Li in which Swimming Blastulæ of Arbacia Can Be Obtained.

K mixture.	Natural sea water.	Li mixture.
cc.	*cc.*	*cc.*
0.0	23.0	2.0
0.5	19.5	5.0
1.0	18.0	6.0
2.0	16.0	7.0
4.0	12.0	9.0
6.0	6.0	13.0

against Li 600 per cent by increasing simultaneously the amount of K normally present in the sea water by 1,300 per cent. Since it was necessary to keep all the other constituents of the sea water constant the KCl was not added in the form of a pure M/2 solution of this salt but in the form of a mixture of the following composition which we will call the KCl mixture.

100.0 cc. of M/2 KCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $KHCO_3$

Systematic experiments showed that the maximum dose of Li in which the eggs could develop into larvæ increased with the concentration of K added to the sea water. This is indicated in Table III.

The table shows that if we wish to replace in normal sea water more Na by Li we must at the same time also replace an increasing proportion of Na by K.

In order to obtain a more regular curve than expressed in Table III we replaced the natural sea water by a NaCl mixture free from KCl and made up as follows:

100.0 cc. of M/2 NaCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $NaHCO_3$

Experiments were then made to ascertain the maximal amount of Li mixture which permitted the formation of swimming blastulæ for each given amount of KCl. The results are contained in Table IV.

TABLE IV.

Maximal Amount of Li Mixture Permitting Formation of Swimming Blastulæ.

K mixture.	Na mixture.	Li mixture.
cc.	*cc.*	*cc.*
0.1	24.0	1.0
0.2	23.0	2.0
0.5	22.0	3.0
1.0	20.0	4.0
2.0	17.0	6.0
4.0	13.0	8.0
6.0	10.0	9.0
7.0	8.0	10.0

When 8 cc. of K mixture were used no more larvæ were obtained on account of the fact that this concentration of K itself was too toxic.

Table IV shows more clearly than Table III that by replacing more Na ions by K ions we increase at the same time the proportion of Na ions which can be replaced by Li ions, without preventing the development of the sea urchin egg into a swimming larva.

It was then shown that Rb has a similar but not quite so great an effect as K (Table V). RbCl was also added in the form of a mixture containing all the other constituents of sea water except K and Na.

Cs acts also somewhat like K but still less weakly than Rb. Only one series of experiments was made in an attempt to replace K by Cs, and this series proved that in 2 cc. of M/2 CsCl mixture + 19 cc. of natural sea water + 4 cc. of M/2 Li mixture swimming larvæ could be obtained. When no sea water was replaced by CsCl, 2 cc. of the Li mixture in 25 cc. was the maximum which still permitted the development of larvæ.

These results show that the toxic effects of Li, which occupies in the periodic table a position on one side of NaCl, are mitigated by the addition of ions like K, Rb, and Cs, occupying a position on the other side of Na. A mixture of Li and K ions in proper proportions acts more like a solution of Na ions than do the Li ions alone.

TABLE V.

Maximal Amount of Li Mixture Permitting Formation of Swimming Larvæ.

Rb mixture.	Sea water.	Li mixture.
cc.	*cc.*	*cc.*
1.0	20.0	4.0
2.0	18.0	5.0
4.0	14.0	7.0

It should be taken into consideration that in these experiments the balance between monovalent and bivalent cations was not disturbed.

If we replace a smaller or larger percentage of the Na ions contained in sea water by Mg or by Ca ions the toxicity of LiCl is not diminished. This is probably due to the fact that the quantity of Ca or Mg required for balancing the monovalent cations is naturally present in the sea water.

The antagonism between LiCl and KCl can also be demonstrated in the eggs of *Fundulus*. When newly fertilized eggs of *Fundulus* are put into M/5 LiCl all the eggs are dead before an embryo is formed. If, however, the M/5 solution of LiCl also contains a small quantity of RbCl or of KCl, as many as 20 per cent of the eggs live long enough to form embryos. When, however, the M/5 LiCl solution contains NaCl not a single embryo is formed. CsCl also gives a positive effect. The M/5 LiCl solution was made up in distilled water and in different

concentrations of the salts mentioned. Table VI gives the results of an experiment. The first horizontal row gives the molecular concentration of NaCl, KCl, RbCl, and CsCl in which the M/5 solution of LiCl was made up. The figures in the next horizontal rows give the percentage of eggs which formed embryos.

The table shows that the addition of NaCl did not protect the *Fundulus* eggs against the toxic effects of LiCl, while the KCl, RbCl, and CsCl had a protective or antagonistic effect. The protective effect of these salts is considerably less than that produced by a salt with a bivalent cation since in the latter case 80 per cent or more of the eggs form embryos in a M/5 solution of LiCl.

TABLE VI.

	Percentage of eggs which formed embryos in M/5 LiCl made up in					
	0	M/40	M/20	M/10	M/5	3 M/10
NaCl	0	0	0	0	0	0
KCl	0	18	10	14	11	6
RbCl	0	7	13	18	20	2
CsCl	0	2	0	5	9	2

These examples may suffice to show that the action of the potassium ion in the phenomena of antagonism (which underlie the physiological balance of ions in salt solutions) is in agreement with the purely chemical character of this ion, *i.e.* its position in the periodic table; and that hence there is no reason to attribute its physiological action in these cases to some other factor; *e.g.*, its extremely minute radioactivity.

SUMMARY.

1. It is shown that the NH_4 ion acts in cases of antagonism on the egg of *Fundulus* more like the K ion than the Na ion; this corresponds to the fact that in its general chemical behavior the NH_4 ion resembles the K ion more closely than the Na ion.

2. It is shown that the tolerance of sea urchin eggs towards the Li ion can be increased 500 per cent or more if at the same time a certain amount of Na ion is replaced by K, Rb, or Cs ions. Since in the

periodic table Na occupies a position between K and Li it is inferred that the Li and K ions deviate in their physiological action in the opposite direction from the Na ion.

3. These data indicate that the behavior of the K ion in antagonistic salt action (which forms the basis of the physiologically balanced action of ions) is due to its purely chemical character, *i.e.* its position in the periodic table or rather to its atomic number, and not to those explosions in its nucleus which give rise to a trace of radioactivity.

ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS. II.

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I. Combining Ratios of Acids and Bases with Gelatin and the Swelling of Gelatin.

In this paper we will continue the demonstration of the relation between the combining ratios of acids and bases with proteins and the effect of ions on the physical properties of proteins. This demonstration completes the proof that the purely chemical forces of primary valency determine the reactions of proteins with other compounds.

It is generally stated in colloidal literature that gelatin swells more in chlorides, bromides, or nitrates than in water and that it swells less in citrates, acetates, tartrates, phosphates, and sulfates than in water. The author of this statement is Hofmeister[1] who was a pioneer in this work and who cannot be blamed for not having considered certain sources of error in his methods. In Hofmeister's experiments gelatin blocks were put into salt solutions of so high a concentration that—as we now know—no specific ion effects could be expected and the slight differences in swelling actually observed by him were probably merely accidental. He even mentions that sugar solutions have a "dehydrating" effect, and this fact alone should have warned chemists against using his experiments for conclusions concerning the specific effects of ions on the physical properties of colloids. As far as the writer can determine from the literature the discrimination between "hydrating" and "dehydrating" ions originated from these experiments.

[1] Hofmeister, F., *Arch. exp. Path. u. Pharmakol.*, 1891, xxviii, 210.

It is often asserted that Hofmeister's ion series for swelling has been confirmed by other authors. Thus Zsigmondy[2] makes the following statements in support of this impression.

"Wo. Ostwald who compared the efficiency of different acids found that swelling diminishes in the acids in the following order,

$$HCl > HNO_3 > \text{acetic acid} > \text{sulfuric acid} > \text{boric acid}.$$

Fischer has shown that the acid and alkali swelling of gelatin as well as that of fibrin is diminished by the addition of salt, and that chlorides, bromides, and nitrates have a less dehydrating action than acetates, sulfates, or citrates. We have here a similar series as in the case of the precipitation of proteins by alkali salts, although the order does not agree entirely."

The writer is inclined to interpret Ostwald's and Fischer's experiments differently from Zsigmondy, since both authors ignored the hydrogen ion concentration of their solutions. We believe to have shown that it is necessary to base conclusions concerning the relative efficiency of ions on experiments with equal hydrogen ion concentration. By ignoring this postulate Ostwald has only succeeded in proving that acetic and boric are weaker acids than nitric but not that gelatin swells less in acetates or borates than in nitrates; and Fischer has only succeeded in proving that citrates and acetates are buffer salts which when added to a solution of a strong acid diminish its hydrogen ion concentration, but not that acetates and citrates diminish the swelling of gelatin. These authors attributed the effects caused by a variation in the hydrogen ion concentration of their solutions erroneously to an influence of the anion. The Hofmeister series of ion effects on swelling has,in reality, never been confirmed.

If we wish to study the specific effects of ions on the swelling of gelatin we must proceed from isoelectric gelatin, bring it to different pH values by different acids or alkalies, and then compare the swelling at the same pH for these different acids or alkalies. If this is done it is found that when gelatin is in combination with the anion of a weak dibasic or tribasic acid, *e.g.* tartaric, citric, phosphoric, its degree of swelling is practically the same as when it is in combination with Cl or NO_3; since in all these cases the anion of the gelatin salts is monovalent. Only in the case of gelatin sulfate is the swelling considerably less,

[2] Zsigmondy, R., Kolloidchemie, Leipsic, 2nd edition. 1918, 373.

because the anion is divalent, H_2SO_4 combining with gelatin in equivalent and not in molecular proportions as do the weak dibasic or tribasic acids; *e.g.*, tartaric or phosphoric.

A few words are necessary concerning the method of these experiments. We can measure the amount of swelling by determining the increase in weight of a given mass of gelatin or by determining its increase in volume. We have adopted the following simple and quick volumetric method (although we intend to supplement these experiments later with gravimetric experiments).

Dry powdered gelatin, of pH = 7.0, was sifted and the grains no longer going through Sieve 50 but going through Sieve 40 or 30 were selected for the experiment. We had therefore fairly uniform grains of not too small a diameter. Doses of 1 gm. each of such powder were weighed out, each dose was put for an hour into 100 cc. of M/128 acetic acid at 10°C. to bring the gelatin to the isoelectric point. The powdered mass was then put on a filter and washed five times with 25 cc. of distilled water at 5°C. In the acetic acid solution and during the washing on the filter the powdered gelatin is stirred constantly.

Each dose of originally 1 gm. of dry powder which had meanwhile absorbed a certain quantity of liquid (which was about the same for each gram of the isoelectric powder) was then put for 1 hour at about 20° into 100 cc. of different concentrations of the acid or base whose influence on swelling was to be tested, and the suspension was constantly agitated. It was found that in an hour the granules of gelatin had reached the maximal swelling in each solution. To measure the relative amount of swelling in different acids or alkalies and at different pH the suspension was poured into graduate cylinders of 100 cc. each (and all of the same diameter) in which the granules fell very rapidly to the bottom. The cylinders were kept in a water bath at 20°C. for about 10 to 15 minutes and the volume occupied by the gelatin granules was then read. This volume included a certain amount of solution between the granules and therefore the real volume of the gelatin was smaller than that read. While therefore the method cannot be used to measure the absolute amount of swelling it allowed us to determine the relative influence of different acids or bases on the swelling for the same pH.

The determination of the pH of the gelatin in these experiments requires a short discussion since the pH inside the gelatin is quite different from that in the supernatant liquid, owing to the Donnan equilibrium. Donnan has shown that when a solution of a colloidal salt is separated from the solution of a crystalloidal salt with a common ion by a membrane which is permeable for the crystalloidal but not for the colloidal ions the concentration of the crystalloidal salt is, at the point of equilibrium, always lower on the side of the colloidal solution than on the side of the crystalloidal solution.[3] This was invariably the case in our experiments on osmotic pressure reported in the preceding paper. When, for example, a gelatin chloride solution of pH 3.5 was put inside a collodion bag and the latter was dipped into a solution of HCl (without gelatin) also of a pH 3.5, the pH on the two sides of the membrane did not remain the same since some of the free acid was forced from the colloidal solution into the pure acid solution outside the collodion bag, so that the pH of the outside solution fell while that of the inside rose.

As Procter[4] has pointed out this Donnan equilibrium must play a rôle also in the case of the swelling of gelatin since in this case the surface of the gelatin granule takes the place of the membrane permeable for the crystalloidal electrolyte but not for the colloid.

In our experiments 1 gm. of originally isoelectric gelatin was put for 1 hour at 20°C. into 100 cc. of acid, *e.g.* HCl, of different concentration varying from M/16 to M/8,192. After an hour equilibrium was reached and the pH of the supernatant fluid was determined. The gelatin was put on a filter (after the volume of the gelatin in the graduate cylinder had been measured) and all the acid was allowed to drain off. A trace of outside acid probably remained on the surface of each granule though presumably some of the free acid inside the granule diffused to the surface under the influence of pressure. This error was partly but not completely compensated by adding enough distilled water of pH of about 5.6 to the gelatin after it had

[3] Donnan, F. G., *Z. Electrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

[4] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

been melted to bring the volume to 100 cc. The pH of the 1 per cent solution of originally isoelectric gelatin was determined colorimetrically. It was found that the pH of the supernatant HCl solution was always considerably smaller than the pH of the gelatin solution (Table I).

The first row in Table I gives the molecular concentration of the 100 cc. of HCl into which the gelatin was originally put. The second row gives the pH of these supernatant HCl solutions after 1 hour, and the third row gives the pH of the gelatin solutions after the supernatant HCl solution had been drained off and after the remaining mass of gelatin had been melted and brought to a volume of 100 cc. by adding enough distilled water of pH 5.6. It will be noticed: first, that the pH of the supernatant HCl solution after 1 hour is higher than the pH of the original HCl solution owing to the fact that some acid combined with the gelatin; and, second, that the pH of the gelatin solution is considerably higher than that of the supernatant solution owing to the Donnan equilibrium, according to which the concentration of free acid outside the gelatin must be greater than inside.

In order to get the correct difference due to the Donnan equilibrium, solutions of gelatin salts were put into collodion bags and these bags were dipped into beakers containing 350 cc. of solution of the same acid or base as that inside the collodion bag and possessing the same pH as the gelatin solution; the outside solution, of course, was free from gelatin. In the case of gelatin-acid salts free acid invariably diffused from the gelatin solution into the pure acid solution, *e.g.* HCl, so that the pH of the latter became smaller and that in the gelatin solution higher. In these experiments the volume of the outside pure HCl solution was 350 cc. and that of the inside 1 per cent gelatin solution only 50 cc. Table II gives the result of one experiment of this kind.

Thus at equilibrium pH the gelatin solution was 3.8 and the outside solution 3.2, or inside 3.3, and outside 4.0. This difference is in the same sense and of nearly but not quite the same order of magnitude as that observed in Table I.

We may therefore conclude that the real pH of the gelatin solution inside the granules of gelatin was slightly less than that measured by our method.

TABLE I.

Original concentration of supernatant HCl solution.	M/16	M/32	M/64	3 M/256	M/128	3 M/512	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192
pH of the supernatant HCl solution after 1 hr..	1.25	1.6	1.9	2.1	2.35	2.55	2.8	3.2	3.4	3.6	3.85	4.0
pH of 1 per cent gelatin solution after 1 hr.....	2.2	2.3	2.7	3.0	3.25	3.4	3.8	4.2	4.6	4.65	4.7	4.7

TABLE II.

pH at beginning of experiment, inside and outside.....	2.3	2.6	3.0	3.1	3.3	3.5	3.6	3.75	4.1	4.38	4.8
pH of outside HCl solution after 20 hrs.....	2.1	2.5	2.9	3.0	3.15	3.2	3.3	3.4	3.7	4.0	4.8
pH of gelatin solution after 20 hrs.....	2.6	3.2	3.3	3.5	3.7	3.8	4.0	4.1	4.2	4.4	4.8

TABLE III.

Original concentration of supernatant KOH solution.....	M/32	M/64	M/128	M/256	M/512	7 M/4,096	6 M/4,096	5 M/4,096	M/1,024	M/2,048	M/4,096	M/8,192
pH of the supernatant KOH solution after 1 hr..	12.4	12.0	11.6	11.3	8.0	7.2	7.0	6.6	6.6	5.8	5.1	5.2
pH of 1 per cent gelatin solution after 1 hr.....	12.0	11.6	11.0	9.8	6.3	5.7	5.5	5.4	5.3	5.1	4.9	4.8

The results of our experiments on swelling are expressed in Figs. 1, 2, and 3. The abscissæ in Fig. 1 are the pH found in the gelatin after equilibrium was established. The ordinates represent the figures for the volume of the granules of 1 gm. of gelatin in different acids. It is obvious that in all cases the volume (or swelling) is a

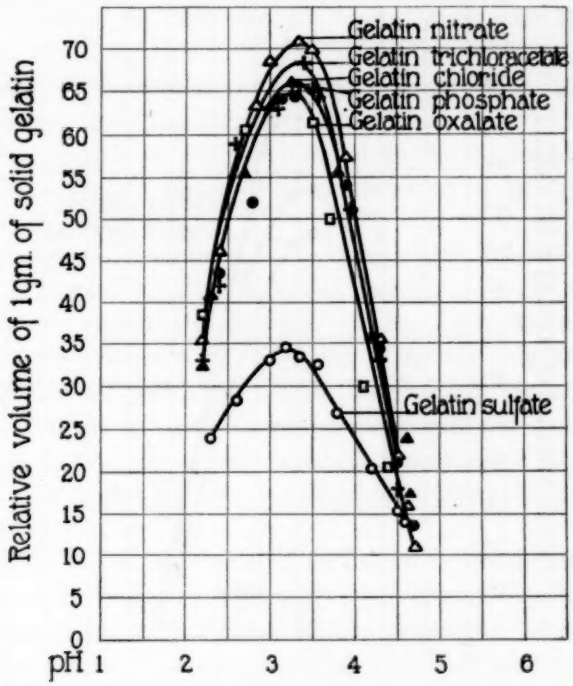

FIG. 1. Influence of HCl, HNO_3, H_3PO_4, H_2SO_4, trichloracetic, and oxalic acids on the swelling of gelatin. Abscissæ are the pH, ordinates the volume of gelatin. The curves for all the acids are practically identical except that for H_2SO_4 which is about one-half as high as the curves for the other acids.

minimum at the isoelectric point pH = 4.7, that it rises with diminishing pH until the maximum is reached at a pH of about 3.2 or 3.3, and that the curve drops steeply with a further diminution of pH (*i.e.* a further increase of hydrogen ion concentration). The fact that the maximum lies here at pH of about 3.2, while in our osmotic pressure curves it was at about 3.3 or 3.4, indicates the degree of error in

the measurement of pH in this case due to the adhesion of some of the original acid on the outside of the granules. This error was partly compensated by the addition of distilled water of pH of about 5.6 in making up the 1 per cent solution of gelatin. On the whole the

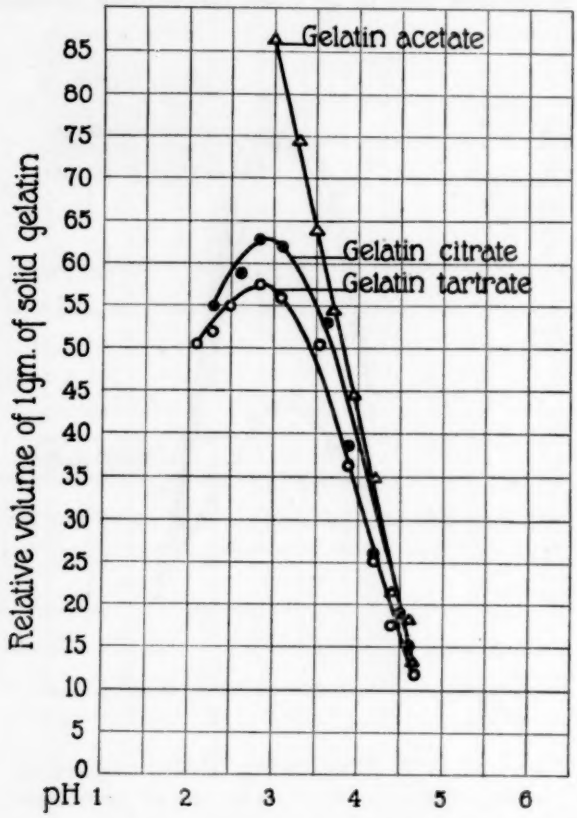

FIG. 2. Influence of citric, tartaric, and acetic acids on swelling of gelatin. The curves for citric and tartaric acids are practically identical with those for HCl and HNO_3 in Fig. 1. That for acetic acid is a little higher owing probably to some specific and secondary effect of this acid.

probable error was +0.1 or +0.2; *i.e.*, the real pH was 0.1 or at the utmost 0.2 greater than in our abscissæ. The most important fact is, however, that the curves for the influence of HCl, HNO_3, trichloracetic, oxalic, phosphoric, citric, and tartaric acids are practically

identical (Figs. 1 and 2), proving that only the effect of the valency and not that of the nature of the anion of the acid used influences the swelling; since we have seen that the anion of weak dibasic or tribasic organic acids combining with the gelatin is always monovalent.

The curve for the swelling of gelatin sulfate, where the anion combining with gelatin is bivalent, is only half as high as the curve for the salts of gelatin with the anion of weak dibasic acids (Figs. 1 and 2).

Acetic acid gives an increasing amount of swelling (Fig. 2), but it must be remembered that M/1 acetic acid had to be used to bring the pH of the gelatin to 3.0, and it is not impossible that in this case a secondary chemical or physical modification of the gelatin may complicate the conditions.

It is of interest to compare these curves with those which should be expected according to the Hofmeister series. In the latter case the curves for phosphate, oxalate, citrate, tartrate, and acetate should coincide with the curve for sulfate instead of coinciding with the curves for Cl and NO_3. This difference is due to the fact that the believers in the Hofmeister series did not determine the pH and that they erroneously ascribed the effects due to a variation in the hydrogen ion concentration to a difference in the influence of the anion.

The ratio between the effects of sulfuric acid on swelling and that of the other acids is again not far from 1:2. If we deduct the swelling of the isoelectric gelatin (of about 10 mm.) from the values of our ordinates the swelling of gelatin sulfate at pH of about 3.3 is less than one-half that of the other gelatin-acid salts where the anion in combination with gelatin is monovalent.

When powdered isoelectric gelatin is treated with an alkali, *e.g.* KOH, the supernatant watery solution is less acid or more alkaline than the gelatin granules. The CO_2 of the air lowers the pH of the solutions a little but this error affects the pH of the supernatant watery solution more than it does the gelatin which has a buffer action. Table III, p. 252, gives the original concentration of the watery solution of KOH, into 100 cc. of which 1 gm. of powdered isoelectric gelatin was put (first row, Table III). After 1 hour the pH of the supernatant watery solution was determined (second row, Table III);

the supernatant solution was drained off from the gelatin, the latter melted, and the volume brought to 100 cc. by adding distilled water of pH of about 5.6, and the pH of the gelatin solution was determined (third row, Table III).

It is obvious that the pH of the supernatant solution is higher than that of the gelatin solution, as we should expect from the Donnan equilibrium.

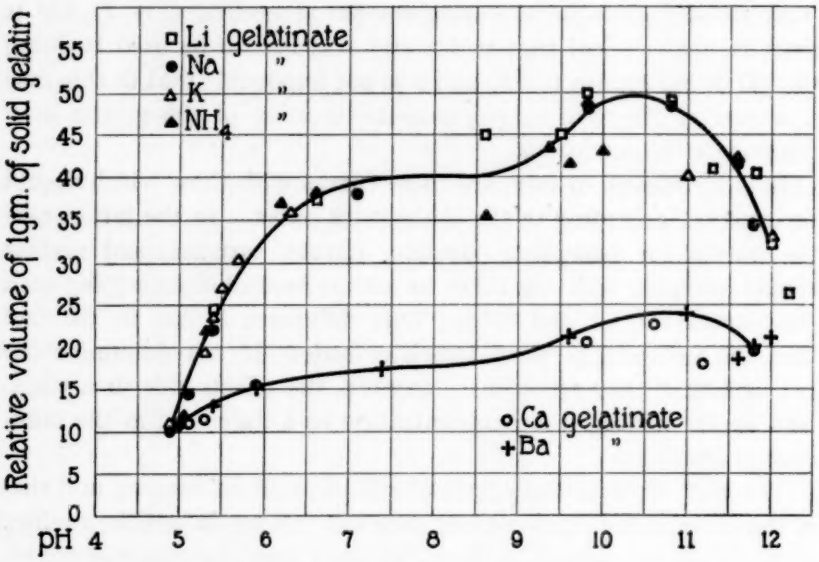

FIG. 3. Curves for the effect of different bases on swelling. Those for LiOH, NaOH, KOH, and NH_4OH are practically identical and about twice as high as those for $Ca(OH)_2$ and $Ba(OH)_2$.

Fig. 3 gives the curves for the action of alkalies on swelling. The curves for Li, Na, K, and NH_4 gelatinate of the same pH are practically identical, except that the values for NH_4OH are irregular for pH above 8.5 possibly on account of the fact that the concentration of NH_4OH required to bring gelatin to such pH is rather high. The main fact is that the ratio of the maximal swelling of gelatin salts with bivalent cation like Ca or Ba is half or possibly a little less than half of that of gelatin salts with monovalent cation, like Na, K, or NH_4.

Near pH = 7.0 the curves run parallel to the axis of abscissæ for the reason that a considerable variation in pH signifies only a negligible change in the concentration of gelatin salt formed. The experiments were not carried beyond a pH of 12.0 on account of the lack of reliable indicators for that region, and on account of the fact that alkali causes chemical changes in the gelatin.

It should be pointed out that the maximal swelling of gelatin in alkalies was less than that in acids. This was not observed in the osmotic pressure curves.

II. Relative Solubility of Different Gelatin Salts in Mixtures of Water and Alcohol.

When powdered gelatin is brought to the isoelectric point, melted, and made into a 1 per cent solution it is at first transparent. After some time, which is the shorter the lower the temperature, the gelatin solution becomes opaque; and in the course of weeks or months it may settle in the form of a precipitate. This, however, does not happen in each case, possibly for the reason that the precipitation will occur only at a very definite pH, while, with a slight deviation from this point in either direction, the result will be only an opacity at room temperature. Raising of the temperature will again result in the clearing of the opacity. The opacity seems therefore to be due to the formation of larger aggregates of protein molecules and these will float as long as they are not too large. The setting of the solution to a gel is a different process from this precipitation since no cloudiness or opacity needs to be connected with this latter phenomenon.

When we add to a freshly prepared solution of isoelectric gelatin only a trace of 95 per cent alcohol the cloudiness which would have formed slowly is noticed at once and if we add a little more alcohol we can produce at once a dense precipitate. In order to standardize the degree of cloudiness produced we add so much 95 per cent alcohol to 10 cc. of a 1 per cent solution of isoelectric gelatin in a test-tube of definite diameter until certain letters become illegible when looked at through the test-tube filled with the gelatin-alcohol-water mixture. Since the addition of alcohol to the watery solution raises the temperature and since this has the tendency to diminish the degree of

opacity of the mixture it was necessary to dip the test-tube in ice water during the process of mixing and keep the gelatin solution approximately at 10°C.

When we prepare gelatin chloride by adding small quantities of HCl to isoelectric gelatin we need the more alcohol the lower the pH and very soon a limit is reached when the addition of 25 cc. or more alcohol no longer brings about any precipitate or even cloudiness. Thus 10 cc. of isoelectric gelatin required 2 cc. of 95 per cent alcohol to bring about that high degree of opacity at which the test letters were no longer legible. When the pH of the gelatin was lowered to 4.55

TABLE IV.

Cc. of 95 per cent alcohol required to bring 10 cc. of 1 per cent gelatin-salt solution to standard opacity.

	pH of gelatin-acid salt.									
	4.6	4.55	4.45	4.4	4.2	3.75	3.3	3.1	2.6	2.1
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Gelatin chloride		5.5	∞	∞	∞	∞	∞	∞	∞	∞
" oxalate	5.3	18.1		∞	∞	∞	∞	∞	∞	∞
" phosphate		28.0		∞	∞	∞	∞	∞	∞	∞
" tartrate		11.6		∞	∞	∞	∞	∞	∞	∞
" succinate		3.0	5.4	∞	∞	∞	∞	∞	∞	∞
" citrate		4.2		∞	∞	∞	∞	∞	∞	∞
" acetate		2.7	20.0	∞	∞	∞	∞	∞	∞	∞
" monochloracetate		4.1	∞	∞	∞	∞	∞	∞	∞	∞
" dichloracetate		5.9	∞	∞	∞	∞	∞	∞	∞	∞
" trichloracetate	2.9			∞	∞	∞	∞	∞	∞	∞
" sulfate		6.8		9.0	11.2	12.8	14.6	14.4	13.2	13.3

by the addition of HCl, 5.5 cc. of alcohol were required for the same degree of opacity. When the pH of the 1 per cent gelatin chloride solution was only a trifle lower, namely 4.50, the addition of 25 cc. of alcohol or more did not suffice for bringing about the degree of opacity required for our test; only a lower degree of turbidity resulted. A gelatin chloride solution of pH 4.45 remained perfectly clear (with a bluish tint) regardless of how much alcohol was added. We may say that gelatin chloride becomes soluble in an alcohol-water mixture containing more than 75 per cent alcohol as soon as its pH is $\gtrless$ 4.45.

It seemed of interest to compare the relative solubility of other gelatin-acid salts with that of gelatin chloride. Table IV gives the

result. The figures indicate the number of cc. of 95 per cent alcohol which when added to 10 cc. of 1 per cent gelatin solution brings about the standard degree of opacity. When the addition of 30 cc. or more alcohol to 10 cc. of the 1 per cent solution of gelatin-acid salt leaves the solution perfectly clear we indicate this by the sign ∞.

The result (which agrees with the results of a previous publication by the writer[5]) is unequivocal: all those gelatin-acid salts in which the anion in combination with gelatin is monovalent can no longer be precipitated by 95 per cent alcohol when the pH is $\lesseqgtr$ 4.4; while the only gelatin-acid salt in combination with a bivalent anion, namely gelatin sulfate, can be precipitated at any pH down to 2.0 (or even below). The relative solubility of gelatin-acid salts in alcohol shows,

TABLE V.

Cc. of 95 per cent alcohol required to bring 10 cc. of 1 per cent gelatin-salt solution to standard opacity.

	pH of metal gelatinate.							
	4.9	5.0	5.4	6.4	9.6	10.2	11.4	12.0
	cc.	cc.	cc.	cc	cc.	cc.	cc.	cc.
Li gelatinate.....	2.3	∞	∞	∞	∞	∞	∞	∞
Na "	2.0		∞	∞	∞	∞	∞	∞
K "	1.9	∞	∞	∞	∞	∞	∞	∞
NH_4 "	1.9	∞	∞	∞	∞	∞	∞	∞
Ca "		2.8	4.4	8.2	8.2	10.5	12.0	7.2
Ba "		2.1	4.2	6.6	7.9	8.1	5.9	5.3

therefore, the same influence of the valency (and lack of influence of the nature of the anion) which we have found in connection with the other properties of proteins like swelling, osmotic pressure, and viscosity.

The same agreement exists in regard to metal gelatinates. 10 cc. of a 1 per cent solution of Li, Na, K, and NH_4 gelatinate can no longer be precipitated by the addition of 95 per cent alcohol when the pH is $\gtreqless$ 5.0. The deviation from the isoelectric point is minute. 10 cc. of 1 per cent Ba and Ca gelatinate, however, can be precipitated with comparatively small quantities of 95 per cent alcohol at any pH (Table V).

[5] Loeb, J., *J. Biol. Chem.*, 1918, xxxiv, 489.

We should expect that when the hydrogen ion concentration of a gelatin chloride solution becomes very high its solubility in an alcohol-water mixture will be diminished again. This is indeed the case, and happens when in 100 cc. of 2 per cent solution of isoelectric gelatin are contained 30 or 40 cc. of M/1 HCl. When to 5 cc. of such a solution are added 25 or 20 cc. of 95 per cent alcohol, the turbidity occurs again. When 100 cc. of the solution contain 50 cc. of M/1 HCl only 14.7 cc. of 95 per cent alcohol are required.

The same result was obtained with Na gelatinate which can also be precipitated again by alcohol when its pH exceeds 12 or 13.

The fact that the gelatin-acid salts (with the exception of gelatin sulfate) become completely soluble in alcohol when the pH reaches the low value of 4.4 is not easy to harmonize with the hypothesis of Pauli that this is due to the ionization of the gelatin, since the relative amount of ionized gelatin is exceedingly small at pH 4.4.

The experiments on the relative solubility of different gelatin salts therefore show the same influence of the valency of the ion in combination with gelatin as was shown in regard to the other physical properties of proteins.

III. Conductivity and Ionization of Gelatin Solutions.

The influence of ions on the conductivity of protein solutions should run parallel to the influence on swelling, viscosity, and osmotic pressure, if it be true that these properties depend on the concentration of the protein ions in the solution. According to this theory, first proposed by Laqueur and Sackur[6] and elaborated by Pauli,[7] the values for the physical properties of proteins are a minimum at the isoelectric point for the reason that the ionization of the protein molecules is a minimum at that point. When we add acid, *e.g.* HCl, protein chloride is formed which is highly ionized and the increase in the viscosity, swelling, and osmotic pressure with the increase of acid is explained by the ionization theory on the assumption of an increase in the concentration of the protein ions in the solution. When, however, too much acid is added, *i.e.* as soon as the pH of the gelatin solution

[6] Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

[7] Pauli, W., Kolloidchemie der Eiweisskörper, pt. 1, Dresden and Leipsic, 1920.

falls below 3.3, the swelling, osmotic pressure, and viscosity of the solution diminish again upon the addition of further acid. This would be explained by the ionization theory on the assumption that the concentration of ionized protein in the solution reaches a maximum at a pH of about 3.3, and that a further increase of acid lowers the concentration of ionized gelatin in the solution. The same theory should also explain the fact that the curves for the physical properties of gelatin salts with a bivalent ion are so much lower than the gelatin salts with a monovalent ion by the assumption that the latter are more highly ionized than the former.

We can determine the concentration of ionized gelatin in solution with the aid of conductivity measurements of the solution of a gelatin salt, *e.g.* gelatin chloride, if we deduct the conductivity of the free HCl in the solution from the total conductivity of the gelatin solution, since our gelatin solutions contain no other electrolyte except the free acid, *e.g.* HCl, and the gelatin salt; *e.g.* gelatin chloride. This is proved by the fact that at the isoelectric point our gelatin solutions had practically the conductivity zero (Figs. 4, 6, 8, and 9). Our method of procedure was as follows: doses of 1 gm. of powdered gelatin were brought to the isoelectric point and to each gram of isoelectric gelatin were added different quantities of 0.1 N acid or alkali and some water; the mass was melted by heating to 40° and then so much H_2O was added that the volume of the solution was 100 cc. After that the pH of the gelatin solution and the conductivities were determined.

Fig. 4 gives the curves for such measurements in the case of gelatin chloride. The abscissæ are the pH, the ordinates the specific conductivities multiplied by 10^4. The curve to the right is the total specific conductivity $\times$ 10^4 of the gelatin chloride solution of different pH. The curve to the left represents the measurements of the specific conductivities $\times$ 10^4 of pure HCl solutions (without gelatin) for different pH. By deducting the ordinates of this latter curve from the ordinates of the curve for total conductivity we get the curve in the middle representing the specific conductivity $\times$ 10^4 of the pure gelatin chloride solution. Since it had been shown before that the viscosity of the solution does not influence the conductivity in this case (Hardy,

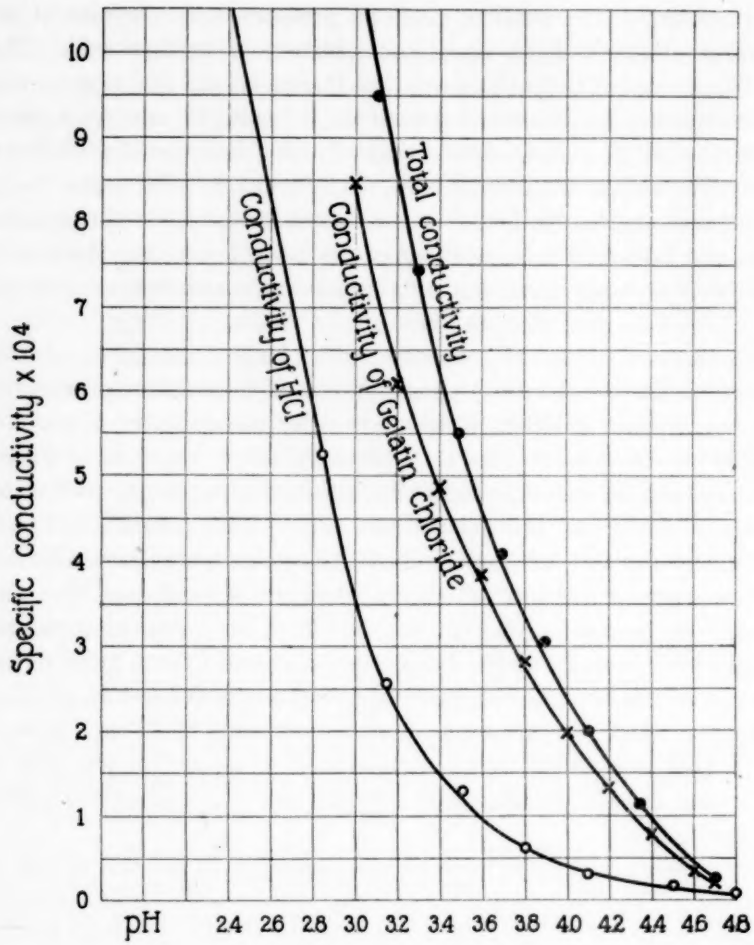

FIG. 4.

FIGS. 4 and 5. Specific conductivity of gelatin chloride solutions of different pH (but all 1 per cent in regard to isoelectric gelatin). Abscissæ are the pH, ordinates specific conductivity $\times 10^4$. Total conductivity means specific conductivity $\times 10^4$ of the gelatin solution measured directly. From this is to be deducted the specific conductivity of HCl of the same pH as the gelatin solution, to obtain the real curve for the specific conductivity of gelatin chloride.

Loeb, Northrop[8]), we may conclude that the middle curve represents the specific conductivity of the gelatin chloride solution and that it

[8] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 605.

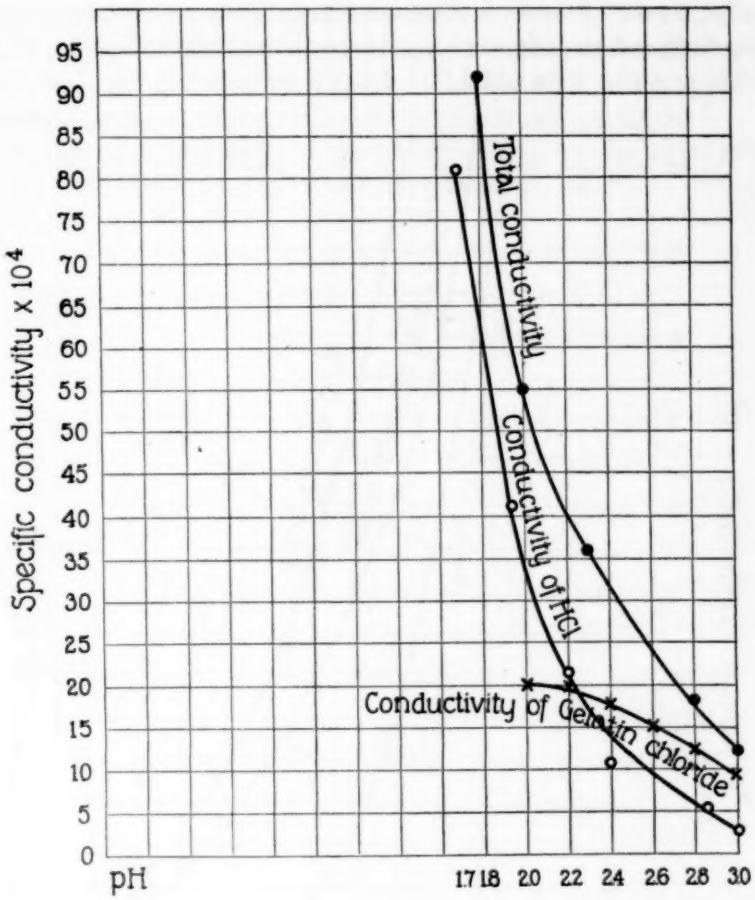

FIG. 5.

can hence be used as a measure of the concentration of the ionized gelatin in the solution. Fig. 4 shows that the curve for the conductivity of gelatin chloride rises continually with increasing hydrogen ion concentration. Fig. 5 is a completion of Fig. 4 for pH down to 2.0. (The ordinates are on a smaller scale in Fig. 5 than in Fig. 4.) It is obvious that at no time does the conductivity curve for gelatin chloride, *i.e.* the curve representing the concentration of ionized protein, show the drop observed in the curves representing the other properties of proteins.

Figs. 6 and 7 show that the same is true for the conductivity curve for gelatin sulfate; Fig. 6 gives the specific conductivities for pH 4.7 to 3.0, and Fig. 7 for pH 3.0 to 2.2 (the ordinates in Fig. 7 are on a

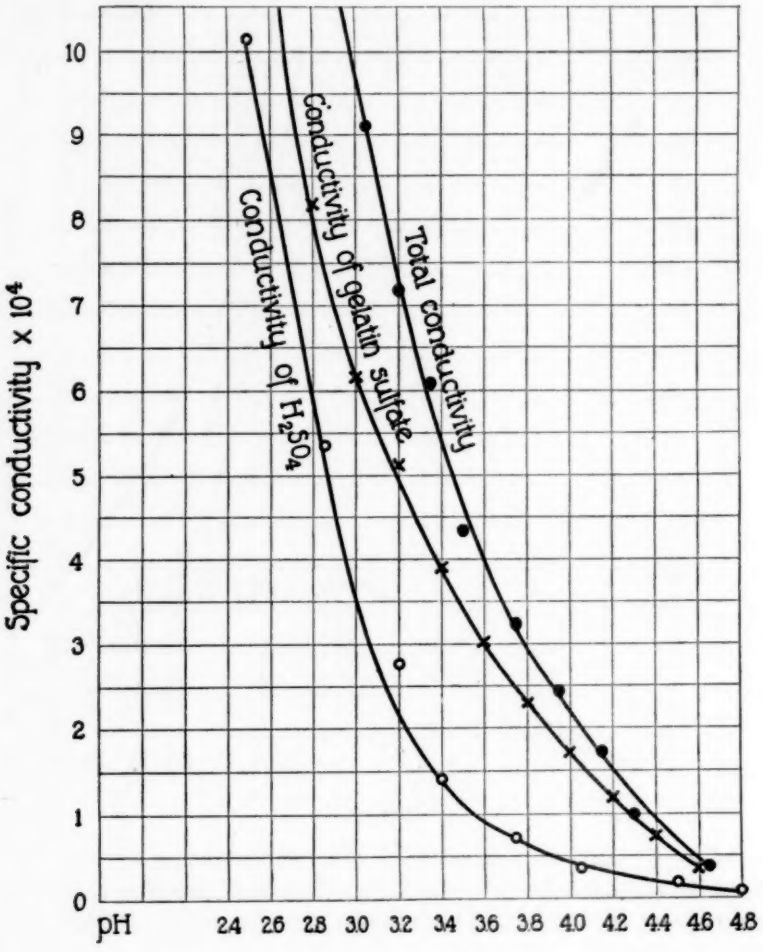

FIG. 6.

FIGS. 6 and 7. Conductivity curves for gelatin sulfate. See legend for Figs. 4 and 5.

smaller scale than in Fig. 6). Experiments on the conductivity of gelatin acetate, trichloracetate, phosphate, and oxalate all give a

similar result. These experiments do not support the hypothesis that the drop in the curves for viscosity, swelling, and osmotic pressure of gelatin-acid salts at or near pH 3.3 is due to a corresponding drop in the degree of ionization of the gelatin salts mentioned.

No drop was discovered in the conductivity curves for metal gelatinates (Na gelatinate, Fig. 8, and Ba gelatinate, Fig. 9).

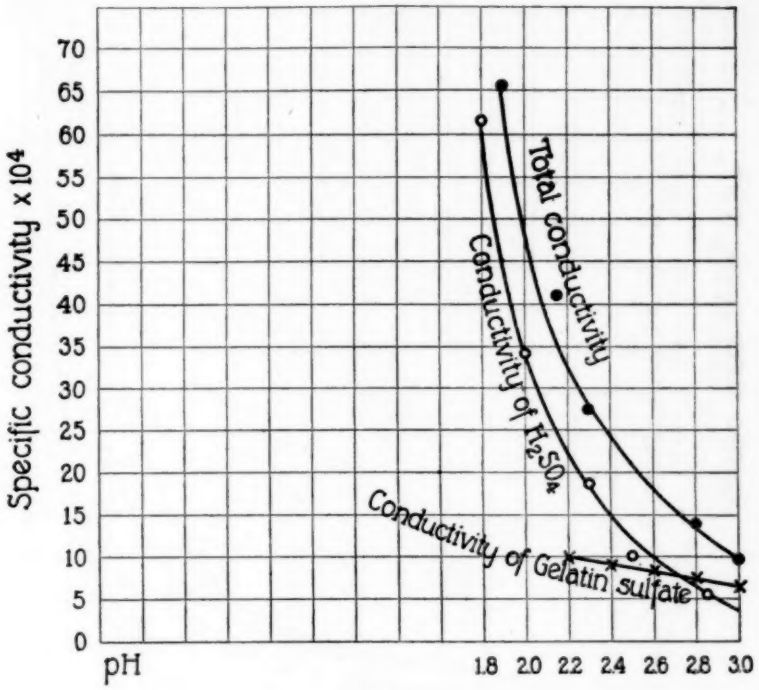

FIG. 7.

The question now arises whether we can explain the difference in the swelling, osmotic pressure, and viscosity of gelatin sulfate on the one hand and gelatin chloride and oxalate, etc., on the other hand on the basis of the ionization theory. If the ionization theory is correct the conductivity of gelatin oxalate, and of gelatin chloride should be twice or almost two and one-half times as great as that of gelatin sulfate. Yet Table VI shows that there is very little difference

between the conductivities of gelatin oxalate and gelatin sulfate; and also a difference of only 20 per cent between gelatin sulfate and gelatin chloride at pH 3.7. As a matter of fact the difference in conduc-

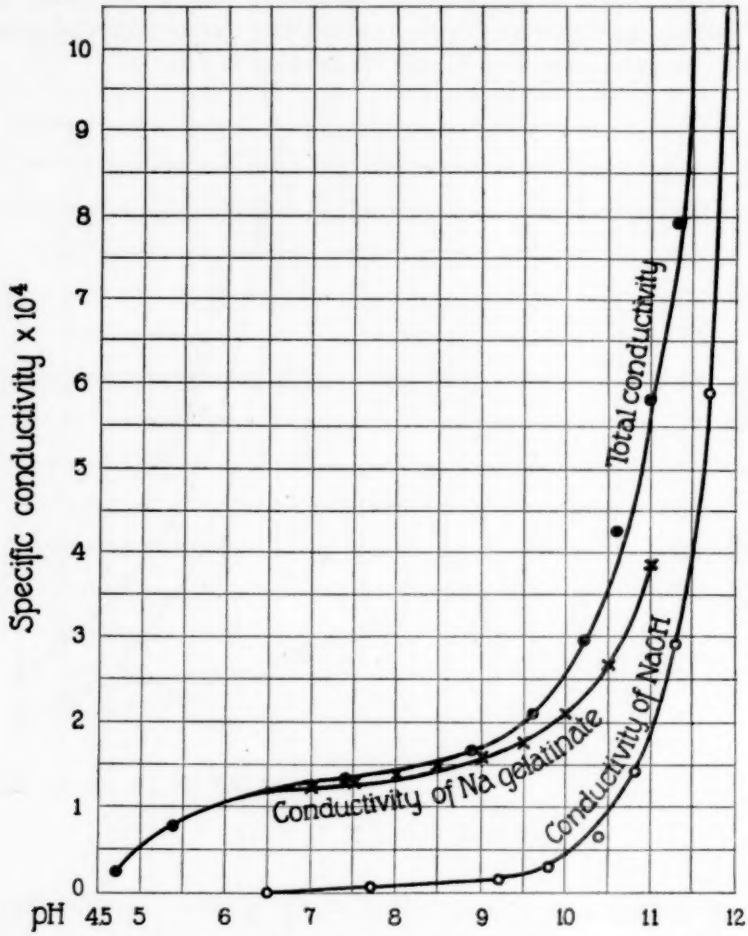

FIG. 8. Conductivity curve for Na gelatinate.

tivity between gelatin oxalate and gelatin chloride which show equal swelling, viscosity, and osmotic pressure is greater than the difference in conductivity between gelatin chloride and gelatin sulfate which are so enormously different in regard to swelling, osmotic pressure, etc.

The three salts, gelatin chloride, sulfate, and oxalate were chosen, since the ionic mobilities of Cl, $\frac{1}{2}$ SO_4, and $\frac{1}{2}$ oxalate are so nearly alike.

It had been pointed out by the writer in a previous paper that the difference in conductivities of Na and Ba gelatinate and of gelatin

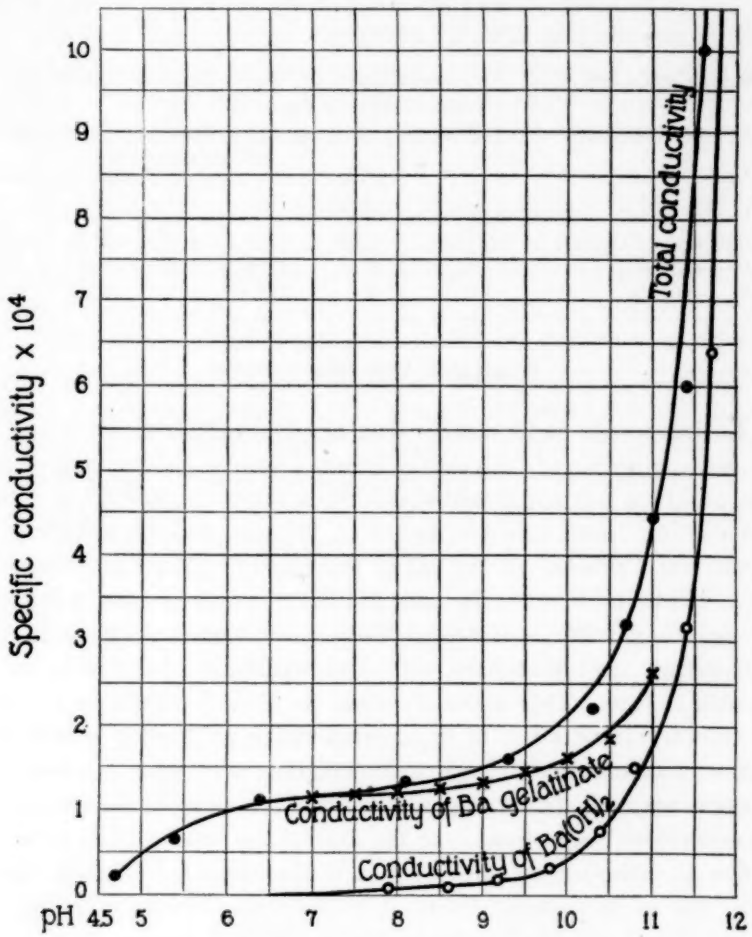

FIG. 9. Conductivity curve for Ba gelatinate.

bromide and gelatin sulfate is too small to account for the difference in the osmotic pressure of solutions of these two types of gelatin salts on the basis of differences in the ionization of the two protein salts.[9]

[9] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483, 569.

These data lend no support to the assumption that the difference between the swelling, viscosity, and osmotic pressure of gelatin sulfate on the one hand, and of gelatin chloride and gelatin oxalate on the other is due to differences in the degree of ionization of proteins.

TABLE VI.

Specific Conductivity of 1 per cent Solutions of Gelatin Chloride, Gelatin Sulfate, and Gelatin Oxalate.

	pH of gelatin-acid salt.				
	4.4	4.0	3.7	3.4	3.0
Gelatin oxalate	0.65	1.45	2.15	3.15	5.25
" sulfate	0.75	1.75	2.60	3.95	6.15
" chloride	0.8	2.0	3.25	4.85	8.5

SUMMARY AND CONCLUSIONS.

1. Our results show clearly that the Hofmeister series is not the correct expression of the relative effect of ions on the swelling of gelatin, and that it is not true that chlorides, bromides, and nitrates have "hydrating," and acetates, tartrates, citrates, and phosphates "dehydrating," effects. If the pH of the gelatin is taken into considertion, it is found that for the same pH the effect on swelling is the same for gelatin chloride, nitrate, trichloracetate, tartrate, succinate, oxalate, citrate, and phosphate, while the swelling is considerably less for gelatin sulfate. This is exactly what we should expect on the basis of the combining ratios of the corresponding acids with gelatin since the weak dibasic and tribasic acids combine with gelatin in molecular proportions while the strong dibasic acid H_2SO_4 combines with gelatin in equivalent proportions. In the case of the weak dibasic acids he anion in combination with gelatin is therefore monovalent and in the case of the strong H_2SO_4 it is bivalent. Hence it is only the valency and not the nature of the ion in combination with gelatin which affects the degree of swelling.

2. This is corroborated in the experiments with alkalies which show that LiOH, NaOH, KOH, and NH_4OH cause the same degree of swelling at the same pH of the gelatin solution and that this swell-

ing is considerably higher than that caused by $Ca(OH)_2$ and $Ba(OH)_2$ for the same pH. This agrees with the results of the titration experiments which prove that $Ca(OH)_2$ and $Ba(OH)_2$ combine with gelatin in equivalent proportions and that hence the cation in combination with the gelatin salt with these two latter bases is bivalent.

3. The fact that proteins combine with acids and alkalies on the basis of the forces of primary valency is therefore not only in full agreement with the influence of ions on the physical properties of proteins but allows us to predict this influence qualitatively and quantitatively.

4. What has been stated in regard to the influence of ions on the swelling of the different gelatin salts is also true in regard to the influence of ions on the relative solubility of gelatin in alcohol-water mixtures.

5. Conductivity measurements of solutions of gelatin salts do not support the theory that the drop in the curves for swelling, osmotic pressure, or viscosity, which occurs at a pH 3.3 or a little less, is due to a drop in the concentration of ionized protein in the solution; nor do they suggest that the difference between the physical properties of gelatin sulfate and gelatin chloride is due to differences in the degree of ionization of these two salts.